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D-5000 Köln 1(DE)**(54) **Glycosylated FGF and production thereof.**

(57) Disclosed are (1) a mutein of a fibroblast growth factor (FGF), the DNA having introduced therein at least one nucleotide sequence coding for a glycosylation site, (2) a DNA coding for the mutein of (1), (3) a vector containing the DNA of (2), (4) a transformant transformed with the vector of (3), and (5) a process for producing the mutein which comprises cultivating in a culture medium the transformant of a yeast or animal cell transformed with a vector of (3), and producing and accumulating the mutein of (1) in the culture medium, whereby the FGF mutein into which at least one glycosylation site has been introduced is improved in productivity, stability and activities, and advantageously used as medicine.

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## GLYCOSYLATED FGF AND PRODUCTION THEREOF

BACKGROUND OF THE INVENTION

The present invention relates to a mutein of a fibroblast growth factor (hereinafter also referred to as FGF) and a technique for producing the same.

5 Fibroblast growth factors (FGFs) are peptide growth factors, which include FGFs basic in isoelectric point (bFGFs) and FGFs acidic in isoelectric point (aFGFs). The whole amino acid sequences of these factors have both been revealed [F. Esch et al., Proc. Natl. Acad. Sci. U.S.A. 85, 6507 (1985) and K. A. Thomas et al., Proc. Natl. Acad. Sci. U.S.A. 82, 6409 (1985)].

10 It has been known that the FGF exhibits growth promoting action on mesoderm-derived cells such as 3T3 cells and vascular endothelial cells *in vitro*, and angiogenic action thereon *in vivo* [D. Gospodarowicz et al., Endocrine Reviews 8, 95 (1987)]. In particular, the angiogenic action of the FGF, together with its cell growth promoting action, is suggestive of a potential therapeutic application for traumas and burns, and as a preventive therapeutic medicine for thrombosis, arteriosclerosis, etc.

The FGFs naturally occur in extremely small quantities, and therefore, it was difficult to collect these. However, producing methods of the FGFs using genetic engineering techniques have recently been developed. The production methods using the genetic engineering techniques are reported in Biochem. Biophys. Res. Commun. 146, 470 (1987); Biotechnology 5, 960 (1987); The Journal of Biological Chemistry 263, 16471 (1988); The Journal of Biological Chemistry 263, 18452 (1988); The Journal of Biological Chemistry 263, 16297 (1988), and the like.

20 As to FGF muteins, a mutein in which Cys residue existing in the FGF is replaced with Ser is described in Biochem. Biophys. Res. Commun. 151, 701 (1988) and The Journal of Biological Chemistry 263, 18452 (1988).

The present inventors presumed that, by modifying the amino acid sequence to introduce a glycosylation site, the FGFs would be improved in stability, intercellular productivity and cell growth promoting activity per molecule, and provided with extracellular secretory activity, and, in addition, that its unidentified biological activities would be activated.

25 There are no data showing that a sugar chain is ligated to the naturally producing FGF. In general, the FGF is considered to be a peptide having no sugar chain. The FGF is a very unstable peptide and easily loses its activities, though it is not confirmed whether or not this is due to the above structure of the FGF. It is essential for the application of the FGFs to medicine and the like to improve stability of FGFs, particularly recombinant FGFs.

30 It has further been proved that a FGF gene has no clear leader sequence, and that most of the FGFs synthesized in cells remain in the cells and a very small amount of the FGFs or no FGFs are secreted outside the cells. No attempts have been successful till now to ligate a typical heterologous leader sequence upstream from the FGF gene to produce and secrete the FGFs. In the production of the FGF by use of a genetic engineering, if the FGF can be secreted outside the cells, it is expected that subsequent purifying processes will be very simplified.

40 SUMMARY OF THE INVENTION

In order to modify sugar chain-free, biologically active proteins such as recombinant FGFs so as not to affect adversely their activities, but to stabilize the proteins to prolong clearance time in blood, and further to secrete FGFs outside the recombinants on production thereof, the present inventors intended to introduce a glycosylation site in the FGFs.

45 The present inventors constructed bFGF muteins modified so as to introduce a glycosylation site by recombinant DNA techniques and site-directed mutagenesis, and investigated improvements in stability, productivity, extracellular secretion and activities, and changes in biological activities. As a result, the present inventors discovered a mutein which could attain these objects, and further investigated based on this information, thus completing the present invention.

The present invention provides:

(1) a mutein of a fibroblast growth factor (FGF) into which at least one glycosylation site has been introduced,

(2) a DNA coding for the mutein of the above item (1),

(3) a vector containing the DNA of the above item (2),

- (4) a transformant transformed with the vector of the above item (3), and  
 (5) a process for producing the mutein of the above item (1) which comprises cultivating a yeast or animal cell transformant transformed with a vector of the above item (3) in a culture medium, and producing and accumulating the mutein of the above item (1) in a culture.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1 is a schematic representation showing the construction of plasmid pALFA310T obtained in  
 10 Example 1;  
 Fig. 2 is a schematic representation showing the construction of plasmid pTB1011 obtained in Example 2(1);  
 Fig. 3 shows a DNA sequence coding for a bFGF mutein contained in the plasmid pTB1011 obtained in Example 2(1), and an amino acid sequence of the mutein for which it codes;  
 15 Fig. 4 is a schematic representation showing the construction of plasmid pTB1012 obtained in Example 2(2);  
 Fig. 5 shows a DNA sequence coding for a bFGF mutein contained in the plasmid pTB1012 obtained in Example 2(2), and an amino acid sequence of the mutein for which it codes.  
 Fig. 6 is a schematic representation showing the construction of plasmid pTB1130 obtained in  
 20 Example 4;  
 Fig. 7 is a schematic representation showing the construction of plasmid pTB1131 obtained in Example 4;  
 Fig. 8 shows a DNA sequence coding for a bFGF mutein contained in the plasmid pTB1130 obtained in Example 4, and an amino acid sequence of the mutein for which it codes;  
 25 Fig. 9 shows a DNA sequence coding for a bFGF mutein contained in the plasmid pTB1131 obtained in Example 4, and an amino acid sequence of the mutein for which it codes;  
 Fig. 10 is a schematic representation showing the construction of plasmid pTB1172 obtained in Example 6; and  
 Fig. 11 shows a DNA sequence coding for a bFGF mutein CN23 contained in the plasmid pTB1172  
 30 obtained in Example 6, and an amino acid sequence of the mutein for which it codes;  
 Fig. 12 is a electrophoresis diagram analyzing the product of transformant *S. cerevisiae* TB39<sup>p-</sup>/pTB1130 in Example 7;  
 Fig. 13 is a electrophoresis diagram analyzing the product of transformant *S. cerevisiae* TB39<sup>p-</sup>/pTB1131 in Example 8;  
 35 Figs. 14 and 15 are schematic representation showing the construction of plasmids pTB1141 and pTB1162 respectively in Example 9; and  
 Figs. 16 and 17 are graphs comparing stability of a mutein of the present invention with those of bFGF and other muteins in Example 10.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present invention, as FGFs before mutagenesis, any FGFs including acidic FGF and basic FGF derived from warm-blooded mammals may be used.

Typical examples thereof include human, bovine and rat bFGFS.

- 45 More specifically, polypeptides containing an amino acid sequence represented by the following formula [I] are preferred:

Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro [I]

Further, polypeptides represented by the following formula [II] are preferred:

- Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-  
 50 Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-  
 Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-  
 Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Va-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-  
 Asn-Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Y -Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-  
 Z -Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser [II]

- 55 wherein Y is Thr or Ser; when Y is Thr, Z is Ser; and when Y is Ser, Z is Pro.

Furthermore, human bFGFs containing an amino acid sequence represented by the following formula [III] are preferred:

Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-

Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Thr-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-  
 5 Gly-Ser-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser [III]

Still further, rat FGFs containing an amino acid sequence represented by the following formula [IV] are preferred:

Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-Lys-Ser-Asp-Pro-His-Val-  
 10 Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Glu-Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Ser-Lys-Thr-Gly-Pro-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser [IV]

Examples of aFGFs include human-derived and bovine-derived ones.

15 As amino acid sequences of bovine aFGFs, one represented by the following formula [V] is preferred: Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr-Cys-Ser-Asn-Gly-Gly-Tyr-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly-Thr-Val-Asp-Gly-Thr-Lys-Asp-Arg-Ser-Asp-Gln-His-Ile-Gln-Leu-Gln-Leu-Cys-Ala-Glu-Ser-Ile-Gly-Glu-Val-Tyr-Ile-Lys-Ser-Thr-Glu-Thr-Gly-Gln-Phe-Leu-Ala-Met-Asp-Thr-Asp-Gly-Leu-Leu-Tyr-Gly-Ser-Gln-Thr-Pro-Asn-Glu-Glu-Cys-Leu-Phe-Leu-Glu-Arg-Leu-Glu-Glu-Asn-His-Tyr-Asn-Thr-Tyr-Ile-Ser-Lys-Lys-  
 20 His-Ala-Glu-Lys-His-Trp-Phe-Val-Gly-Leu-Lys-Lys-Asn-Gly-Arg-Ser-Lys-Leu-Gly-Pro-Arg-Thr-His-Phe-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Leu-Pro-Val-Ser-Ser-Asp [V]

Further, as amino acid sequences of human aFGFs, one represented by the following formula [VI] is preferred:

Phe-Asn-Leu-Pro-Pro-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr-Cys-Ser-Asn-Gly-Gly-His-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly-Thr-Val-Asp-Gly-Thr-Arg-Asp-Arg-Ser-Asp-Gln-His-Ile-Gln-Leu-Gln-Leu-Ser-Ala-Glu-Ser-  
 25 Val-Gly-Glu-Val-Tyr-Ile-Lys-Ser-Thr-Glu-Thr-Gly-Gln-Tyr-Leu-Ala-Met-Asp-Thr-Asp-Gly-Leu-Leu-Tyr-Gly-Ser-Gln-Thr-Pro-Asn-Glu-Glu-Cys-Leu-Phe-Leu-Glu-Arg-Leu-Glu-Glu-Asn-His-Tyr-Asn-Thr-Tyr-Ile-Ser-Lys-Lys-His-Ala-Glu-Lys-Asn-Trp-Phe-Val-Gly-Leu-Lys-Lys-Asn-Gly-Ser-Cys-Lys-Arg-Gly-Pro-Arg-Thr-His-Tyr-Gly-Gln-Lys-Ala-Ile-Leu-Phe-Leu-Pro-Leu-Pro-Val-Ser-Ser-Asp [VI]

30 As the FGFs, human basic FGFs are particularly preferable.

The mutein of the present invention has had introduced at least one glycosylation site. The amino acid sequence of the original peptide or protein may be mutagenized. Such mutagenesis includes, for example, addition of an amino acid(s), deletion of a constituent amino acid(s) and substitution of a constituent amino acid(s) for another amino acid(s).

35 The above glycosylation sites include a site in which an amino acid sequence constituting the glycosylation site is represented by the following formula:

Asn-X-Y

wherein X may be any amino acid residue, and Y is Thr, Ser or Cys.

Specifically, any glycosylation site may be used, as long as it produces an amino acid sequence  
 40 represented by Asn-X-Thr, Asn-X-Ser or Asn-X-Cys (wherein X may be any amino acid) in the molecule. It has been known that some kind of regularity exists for the site of a protein to which a glycosyl chain is ligated in a glycoprotein. Namely, the glycosyl chain is ligated to the Asn residue of the protein. The amino acid sequence containing this Asn residue is a sequence called asparagine sequon, which is represented by a sequence consisting of the above three amino acids. These are described in FEBS Letters 108, 341  
 45 (1979), Biochem. J. 203, 761 (1982), Biochem. J. 209, 331 (1983) and FEBS Letters 96, 179 (1987)

X is preferably an amino acid other than Pro, and more preferably Gly, Tyr, Arg, Ser, Lys, Val or Ala and more preferably Gly, Lys, Val or Ala. Y is preferably Thr or Ser.

The sugar which is added to a FGF mutein according to the present invention may be any one found in known glycosylated proteins. Examples of such sugars include N-acetyl glycosamine, N-acetyl galactosamine, mannose, galactose, fucose and sialic acid.  
 50

The number of sugars in a glycosyl chain is preferably at least one, and more preferably 10 to 20.

Such addition of an amino acid(S) includes addition of at least one amino acid.

Such deletion of a constituent amino acid(s) includes deletion of at least one FGF-constituent amino acid.

55 Such substitution of a constituent amino acid(s) for another amino acid(s) includes substitution of at least one FGF-constituent amino acid for another amino acid(s).

At least one amino acid in the mutein which has at least one amino acid added to the FGF excludes methionine caused by an initiation codon used for peptide expression, or a signal peptide.

The number of added amino acids is at least one, but it may be any number as long as the characteristics of the FGF are not lost.

As to the number of deleted constituent amino acids in the FGF and the mutein which lacks at least one FGF-constituent amino acid, it may be any number as long as the characteristics of the FGF are not lost.

5 Examples of the deleted constituent amino acids include the 10 residues on the amino terminal side of the human bFGF:

Met-Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser,

the 14 residues on the amino terminal side of the human bFGF:

Met-Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro,

15 the 41 residues on the amino acid terminal side of human bFGF:

1 2 3 40  
Met-Pro-Ala-Leu-....-Val,

20 and the 61 residues on the carboxyl terminal side of the human bFGF:

86 87 145 146  
Lys-Cys- ..... -Lys-Ser.

The muteins which lack at least one constituent amino acid of the bFGF include muteins lacking the 7 to 46 amino acid residues on the carboxyl terminal side of the bFGF.

Preferred examples of such deletions include deletion of the following amino acid sequences of the human bFGF:

Amino acid sequence from amino acid No. 101 on

Amino acid sequence from amino acid No. 105 on

Amino acid sequence from amino acid No. 114 on

Amino acid sequence from amino acid No. 118 on

Amino acid sequence from amino acid No. 123 on

Amino acid sequence from amino acid No. 129 on

Amino acid sequence from amino acid No. 137 on

Amino acid sequences from amino acid (not yet) ...

As to the number of the FGF-constituent amino acids before substitution in the mutein which has at least one FGF-constituent amino acid substituted for another amino acid(s), it may be any number as long as the characteristics of the FGF are not lost.

Examples of the constituent amino acids before substitution include cysteine and amino acids other than cysteine. In particular, cysteine is preferable. The amino acids other than cysteine as the constituent amino acids before substitution include aspartic acid, arginine, glycine and valine.

45 When the constituent amino acid before substitution is cysteine, for example, neutral amino acids are preferred as the substituted amino acids. Specific examples of the neutral amino acids include glycine, valine, alanine, leucine, isoleucine, tyrosine, phenylalanine, histidine, tryptophan, serine, threonine and methionine. In particular, serine and threonine are preferable.

When the constituent amino acid before substitution is an amino acid other than cysteine, there are selected as the other substituted amino acids, for example, amino acids different in hydrophilicity, hydrophobicity or electric charge from the constituent amino acid before substitution. Specifically, when the amino acid before substitution is aspartic acid, the substituting amino acids include asparagine, threonine, valine, phenylalanine and arginine, and particularly, asparagine and arginine are preferable.

When the amino acid before substitution is arginine, the substituting amino acids include glutamine, threonine, leucine, phenylalanine and aspartic acid, and particularly, glutamine is preferable.

When the constituent amino acid before substitution is glycine, the substituting amino acids include threonine, leucine, phenylalanine, serine, glutamic acid and arginine, and particularly, threonine is preferable.

When the constituent amino acid before substitution is serine, the substituting amino acids include methionine, alanine, leucine, cysteine, glutamine, arginine and aspartic acid, and particularly, methionine is preferable.

When the constituent amino acid before substitution is valine, the substituting amino acids include serine, leucine, proline, glycine, lysine and aspartic acid, and particularly, serine is preferable.

When the constituent amino acid before substitution is isoleucine, the substituting amino acids include serine, glycine, valine, alanine, leucine, tyrosine, phenylalanine, histidine, tryptophan and methionine, and particularly, serine is preferable.

As the original constituent amino acids before substitution, aspartic acid, arginine, glycine, serine and valine are preferred.

As the substituted amino acids, asparagine, glutamine, arginine, threonine, methionine, serine and leucine are preferred.

As the substituted mutein, a mutein in which cysteine, the constituent amino acid, is substituted for serine is most preferred.

Preferred examples of the muteins mutagenized by addition of the amino acid(s), deletion of the constituent amino acid(s) and substitution of the constituent amino acid(s) for another amino acid(s) include bFGF muteins described in Biochemical and Biophysical Research Communications 151, 701-708 (1988), European Patent Publication No. 281,822 and Japanese Patent Application No. 1-15662/1989 (corresponding to European Patent Application No. 89101162.9, Publication No. 326,907).

In the mutein of the present invention, the above mutein having a glycosylation site may further be mutagenized by a combination of at least two of addition, deletion and substitution.

In particular, the above mutein having the glycosylation site in which at least one human bFGF-constituent amino acid is further substituted for another amino acid is preferable. The above mutein having the glycosylation site in which the Cys residues at positions 69 and 87 of the human bFGF are further substituted for Ser residues, respectively, is particularly preferred.

In order to produce the mutein of the present invention, site-directed mutagenesis is employed in addition to the conventional genetic engineering technique. This mutagenesis, which is well known, is described in R. F. Lather and J. P. Lecoq, Genetic Engineering, Academic Press, p.31-50 (1983). Mutagenesis directed to oligonucleotide is described in M. Smith and S. Gillam, Genetic Engineering: Principles and Methods, Plenum Press, vol.3, p.1-32 (1981).

A structural gene coding for the mutein of the present invention is produced, for example, by the steps of:

(a) hybridizing a single-stranded DNA comprising one strand of a structural gene of the FGF with a mutagenic oligonucleotide primer,

(b) elongating the primer with DNA polymerase to form a mutational heteroduplex, and

(c) replicating this mutational heteroduplex.

The size of the oligonucleotide primer depends on conditions required for stable hybridization of the primer to a gene region to which mutation is to be introduced, and on limitations in currently available methods of oligonucleotide synthesis. The factors (for example, the overall size and the size of a mismatching portion for a mutation site) to be considered in designing the oligonucleotide used for mutagenesis directed by the oligonucleotide are described by M. Smith and S. Gillam in the above literature. In general, the overall length of the oligonucleotide is such a length that stable, unique hybridization at the mutation site is optimized, and the extensions from the mutation site to the 5'- and the 3'-termini is adjusted in size so as to be sufficient to prevent mutation editing due to the exonuclease of DNA polymerase. The oligonucleotides used for mutagenesis in accordance with the present invention usually contain about 12 to about 24 bases, preferably about 14 to about 20 bases, and more preferably about 14 to about 18 bases. These usually contain at least about 3 bases on the 3'-terminal side from the codon to be changed.

For example, for the purpose of obtaining a mutein to which an amino acid is added, a gene coding for an amino acid sequence to be added is synthesized, or a fragment is formed by digestion with a restriction enzyme, and then insertion or addition thereof to an appropriate site of a FGF gene with DNA ligase is carried out, thereby producing a mutagenic FGF gene. If any suitable restriction enzyme recognition sites are not present in the FGF gene, the restriction enzyme recognition sites may be newly formed by the above site-directed mutagenesis.

For example, for the purpose of obtaining a mutein lacking a FGF-constituent amino acid, three ways are considered as methods for producing a mutagenic FGF gene. The first one is the case of deleting the amino terminus of the FGF. The second one is the case of deleting a central region of the FGF. The third one is the case of deleting the carboxyl terminus of the FGF.

When the amino terminus is deleted, a codon of a gene coding for the carboxyl terminus of the amino acid sequence to be deleted is mutagenized to an ATG codon coding for Met by site-directed mutagenesis, and an appropriate restriction enzyme recognition site is further formed on the 5'-terminal side of the resulting codon to facilitate its ligation to a promoter.

5 When the central region of the amino acid sequence is deleted, a unique restriction enzyme recognition site is formed on each of the 5'-terminal and the 3'-terminal sides of a gene coding for the sequence to be deleted, and the relevant portion is cleaved off by enzyme digestion, followed by re-ligation of the remaining two fragments to construct a desired gene coding for the FGF lacking the amino acid. At this time, it is of course necessary to prevent a reading frame from shifting due to digestion with the restriction

10 enzyme.  
When the amino acid sequence on the carboxyl terminal side is deleted, a codon of a gene coding for an amino acid on the amino terminal side of the sequence to be deleted is mutagenized to a stop codon by site-directed mutagenesis.

For example, for the purpose of obtaining a mutein whose constituent amino acid, cysteine, is substituted, Cys-expression codons are caused to disappear, or site-directed mutagenesis is induced in the Cys-expression codon TGC or TGT, using a synthetic nucleotide primer which changes the codon so as to code for another amino acid, thereby producing a mutagenic FGF gene. For example, in order to change cysteine (position 25) of the human bFGF to serine, the primer is hybridized with a sense chain of the FGF gene. Preferred examples of the nucleotide primers include 5'-CGTTCTTGCTGTAGAGCCGCT-3', wherein the underlined triplet indicates the changed codon.

20 Preferred primers used when cysteine (position 69) is changed to serine include 5'-AACGATTAGCGCTCACTC-3', wherein the underlined triplet indicates the changed codon.

Preferred primers used when cysteine (position 87) is changed to serine include 5'-GTAACAGACTTAGAAGCTAGT-3', wherein the underlined triplet indicates the changed codon.

25 Preferred primers used when cysteine (position 92) is changed to serine include 5'-TCGAAGAAGAAAGACTCATCC-3', wherein the underlined triplet indicates the changed codon.

In Cys (position 25), cysteine changes to serine by T→A conversion of the first base. Further, in Cys (position 69), cysteine changes to serine by T→A conversion of the first base and T→C conversion of the second base. In Cys (positions 87 and 92), cysteine changes to serine by G→C conversion of the second

30 base.  
When a bFGF mutein protein is produced by site-directed mutagenesis, it should be recognized that a plurality of mutations may be induced in the DNA sequence, namely, that the DNA codons corresponding to the amino acids have degenerated.

For example, for the purpose of obtaining a mutein whose constituent amino acid, an amino acid other than cysteine, is substituted for another amino acid, a codon is mutagenized with an oligonucleotide primer in the same manner as with cysteine, thereby producing a mutagenic FGF gene.

However, as is well known to those of ordinary skill in the art, the design of the oligonucleotide primer varies depending on which amino acid is mutagenized.

The primer is hybridized to a single-stranded phage in which a single strand of the FGF gene is cloned, such as M13 [Yanisch-Perron, C. Vieira and J. Messing, *Gene* 33, 103-119 (1985); J. Messing, *Methods in Enzymology* 101, 20-78 (1983)], fd [R. Herrman et al., *Mol. Gen. Genet.* 177, 231 (1980)] or  $\phi$  X 174 [M. Smith and S. Gillam, *Genetic Engineering*, Plenum Press, vol.3, p.1-32 (1981)]. It is observed that the phage can carry both a sense chain and an antisense chain of the gene. When the phage carries the antisense chain, in addition to discrepancy from the codon determining a triplet which has encoded another amino acid, the primer may not be the same as a sense chain region containing a codon to which mutation is to be induced, due to codon degeneracy. Similarly, when a phage carries a sense chain, the primer may not be complementary to the sense chain region containing a codon to which mutation is to be induced, as well as appropriate discrepancy from a triplet which pairs to a codon to be deleted.

The conditions used for hybridization are described by M. Smith and S. Gillam in the above literature. 50 The temperature is usually within the range from about 0 to 70°C, and more generally within the range from about 10 to 50°C. After hybridization, the primer is elongated on a phage DNA by reaction with *Escherichia coli* DNA polymerase I, T4 DNA polymerase, a reverse transcriptase or another suitable DNA polymerase. The resulting dsDNA is converted to a closed circular dsDNA by treatment with a DNA ligase such as T4 DNA ligase. DNA molecules containing single-stranded regions can be decomposed by S1

55 endonuclease treatment.  
The resulting mutational heteroduplex is used for transformation of infectable host organisms or cells. In the replication of the heteroduplex by using the host, progenies are produced from both chains. Following the replication, a mutant gene is isolated from the progeny of the mutant chain, and inserted into an

appropriate vector. The resulting vector is used for transformation of appropriate host organisms or cells.

Then, the phage DNA carrying the mutational gene is isolated, and incorporated into a plasmid.

Examples of the plasmids into which DNAs are incorporated include plasmids derived from *E. coli*, such as pBR322 [Gene 2, 95 (1977)], pBR325 [Gene 4, 121 (1978)], pUC12 [Gene 19, 259 (1982)] and pUC13 [Gene 19, 259 (1982)]; and plasmids derived from *Bacillus subtilis*, such as pUB110 [Biochemical and Biophysical Research Communication 112, 678 (1983)]. However, any other plasmid may be used, as long as it is replicable and maintainable in the host.

Examples of methods for incorporating the phage DNA in the plasmid include the method described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, p.239 (1982).

The cloned gene is ligated downstream from the promoter in a vehicle (vector) suitable for expression, whereby the expression vector can be obtained.

The vectors include the above plasmids derived from *E. coli* (such as pBR322, pBR325, pUC12 and pUC13), plasmids derived from *B. subtilis* (such as pUB110, pTP5 and pC194), plasmids derived from yeast (such as psH19 and psH15), bacteriophages such as phage, and animal viruses such as retroviruses and vaccinia viruses.

The gene has ATG as a translation initiating codon at the 5'-terminus thereof and may have TAA, TGA or TAG as a translation terminating codon at the 3'-terminus. In order to produce a gene product by secretion in a culture medium, it is preferable to ligate a DNA coding for a signal peptide, or a DNA coding for the signal peptide and a DNA coding for a signal peptide-propeptide (prepro region), to the 5'-terminus of the gene. In order to express the gene, the promoter is ligated to the upstream thereof. As the promoter used in the present invention, any promoter is available as long as it is suitable for expression corresponding to the host used for the gene expression.

When the host used for transformation is yeast, it is preferable to use an  $\alpha$ -factor promoter, a PHO5 promoter, a PGK promoter, a GAP promoter, an ADH promoter and the like. In particular, it is preferable that the host in *Escherichia* and the promoter is a trp promoter, a  $\lambda$ PL promoter or T7 promoter.

When the host is an animal cell, there can be used an SV40-derived promoter, a retrovirus promoter and the like, and particularly, the SV40-derived promoter is preferred.

By using the vector containing the recombinant DNA having a nucleotide sequence coding for the mutein thus constructed, a transformant is prepared.

The hosts are preferably yeast and animal cells for obtaining a FGF mutein which has sugar chain.

Examples of the above yeast include *Saccharomyces cerevisiae* AH22R<sup>-</sup>, NA87-11A<sub>p</sub><sup>-</sup>, DKD-5D and TB39<sub>p</sub><sup>-</sup>.

Examples of the animal cells include monkey cells COS-7, Vero, Chinese hamster cells CHO, mouse L cells and human FL cells.

Preferably, it is desirable to use host-vector systems for which it has been revealed to have a group of enzymes participating in sugar addition, specifically the yeast system and the animal cell system.

The transformation of yeasts is carried out, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A. 75, 1929 (1978).

The transformation of animal cells is carried out, for example, according to the method described in Virology 52, 456 (1973).

As the host of the transformant, *Escherichia coli* may be employed for reserve the plasmid coding for the present mutein. Examples of said *E. coli* include *E. coli* DH1 [Proc. Natl. Acad. Sci. USA, 60, 160(1968)-], JM103 [Nucleic acids Research, 9, 309(1981)], JA221 [Journal of Molecular Biology, 120, 517(1978)], HB101 [Journal of Molecular Biology, 41, 459(1969)], C600 [Genetics, 39, 440(1954)] and MM294 [Proc. Natl. Acad. Sci. USA, 73 4174(1976)].

The transformation of the above-mentioned bacteria of the genus *Escherichia* is carried out in accordance with, for example, the methods described in Proc. Natl. Acad. Sci. USA, 69, 2110(1972), Gene, 17, 107(1982), Molecular Cloning, Cold Spring Harbor Laboratories, page 249, 1982.

Thus, the transformant transformed with the vector containing the recombinant DNA having the nucleotide sequence coding for the mutein is obtained.

The mutein is produced by cultivating the transformant in the culture medium.

When the transformant whose host is a yeast is cultivated, there is used, for example, Burkholder minimum medium [K. L. Bostian et al, Proc. Natl. Acad. Sci. U.S.A. 77, 4505 (1980)] as the culture medium. The pH of the medium is preferably adjusted to about 5 to 8. The cultivation is usually carried out at about 20 to 35°C for about 24 to 72 hours, with aeration or agitation if necessary.

When the transformant whose host is an animal cell is cultivated, examples of the culture mediums include MEM medium containing about 5 to 20% fetal calf serum [Science 122 501 (1952)], DMEM medium [Virology 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association 199, 519 (1967)]



and 199 medium [Proceeding of the Society for the Biological Medicine 73, 1 (1950)]. The pH is preferably about 6 to 8. The cultivation is usually carried out at about 30 to 40 °C for about 15 to 60 hours, with aeration or agitation if necessary.

The mutein can be isolated and purified from the above culture, for example, by the following methods.

5 When the mutein is extracted from the cultivated cells, the cells are collected by methods known in the art after cultivation. Then the collected cells are suspended in an appropriate buffer solution containing a protein denaturant such as guanidine hydrochloride to extract the desired protein out of the cells. There is also suitably used the method that the cells are disrupted by French press, ultrasonic treatment, lysozyme and/or freeze-thawing, followed by centrifugation to obtain the mutein. In particular, the method using  
10 lysozyme in combination with ultrasonic treatment is preferable.

The purification of the mutein from a supernatant can be carried out by suitable combinations of known separating and purifying methods per se.

These known separating and purifying methods include methods utilizing solubility such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as  
15 dialysis, ultrafiltration, gel filtration chromatography and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectro focusing electrophoresis.

20 More specifically, the above supernatant is subjected to ion-exchange chromatography having DEAE cellulose as a carrier, and thereby impurities such as nucleic acids and acidic proteins can be removed. For example, it is effective to apply the supernatant to a DEAE cellulose column equilibrated with an approximately neutral buffer solution such as Tris and collect fractions not adsorbed to the column. Further, the supernatant is subjected to ion-exchange chromatography having CM cellulose or the like as a carrier to  
25 cause the mutein to be adsorbed to the carrier, and then the mutein can be eluted with a salt solution. These eluates may be lyophilized after dialysis.

In the above purification, which fractions contain the desired mutein of the present invention is determined by enzyme immuno assay using, for example, anti FGF antibodies, especially anti FGF monoclonal antibodies (for example, those produced by the method described in European Patent  
30 Application Publication No. 288,687).

The mutein of the present invention is improved in productivity on production thereof, and favorable for industrial mass production, because the mutein is secreted out of the transformant.

As to the mutein of the present invention, the stability is improved, the clearance time in blood is prolonged, and further, the activities are improved. The mutein of the present invention can be therefore  
35 advantageously used as pharmaceutical preparations and the like.

The mutein thus obtained has cellular growth promoting activity, and is low in toxicity. It can therefore be used as therapeutic accelerators for burns, wounds, postoperative tissues and the like or as medicine based on its angiogenic action for thrombosis, arteriosclerosis and the like. It can also be used as reagents for promoting cell cultivation.

40 When the mutein of the present invention is used as pharmaceutical preparations, it can be safely administered parenterally or orally to warm-blooded animals (such as human, mouse, rat, hamster, rabbit, dog and cat), in a powder form as it is, or as pharmaceutical compositions (such as injections, tablets, capsules, solutions and ointments) with pharmacologically acceptable carriers, excipients, diluents and the like.

45 The injections are prepared by conventional methods using, for example, physiological saline or aqueous solutions containing glucose or other auxiliary agents. The pharmaceutical compositions such as tablets and capsules can also be prepared in accordance with conventional methods.

When the mutein of the present invention is used as the above pharmaceutical preparations, it is administered, for example, to the above warm-blooded animals in an appropriate amount ranging from  
50 about 1 ng/kg to 100 µg/kg daily, taking into account the route of administration, symptoms, etc.

Further, when the mutein of the present invention is used as the reagents for accelerating cell cultivation, it is preferably added to a culture medium so as to be contained in an amount of about 0.01 to 10 µg per liter of medium, more preferably about 0.1 to 10 µg per liter of medium.

When bases, amino acids and so on are indicated by the abbreviations in this specification and the  
55 drawings, the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the optical isomer is capable of existing with respect to the amino acids, the L-form is represented unless otherwise specified.

- DNA : Deoxyribonucleic acid  
 cDNA : Complementary deoxyribonucleic acid  
 A : Adenine  
 T : Thymine  
 5 G : Guanine  
 C : Cytosine  
 RNA : Ribonucleic acid  
 dATP : Deoxyadenosine triphosphate  
 dTTP : Deoxythymidine triphosphate  
 10 dGTP : Deoxyguanosine triphosphate  
 dCTP : Deoxycytidine triphosphate  
 ATP : Adenosine triphosphate  
 Tdr : Thymidine  
 EDTA : Ethylenediaminetetraacetic acid  
 15 SDS : Sodium dodecyl sulfate  
 Gly : Glycine  
 Ala : Alanine  
 Val : Valine  
 Leu : Leucine  
 20 Ile : Isoleucine  
 Ser : Serine  
 Thr : Threonine  
 Cys : Cysteine  
 Met : Methionine  
 25 Glu : Glutamic acid  
 Asp : Aspartic acid  
 Lys : Lysine  
 Arg : Arginine  
 His : Histidine  
 30 Phe : Phenylalanine  
 Tyr : Tyrosine  
 Trp : Tryptophan  
 Pro : Proline  
 Asn : Asparagine  
 35 Gln : Glutamine

In this specification and the drawings, with respect to the number of the human bFGF-constituent amino acids, Pro of the N-terminus of the amino acid sequence represented by the above formula [III] is taken as the first.

*S. cerevisiae* TB39 $\rho^-$  hereinafter shown in Reference Example and Example 3 has mycological properties similar to those of seed *S. cerevisiae*, shows the phenotype (a, LEU $^-$ , HIS $^-$ ,  $\rho^-$ ) and the genotype ( $\alpha$ , MATa, leu2, his3, pho9,  $\rho^-$ ), and has the property that the expression amount of genes is high.

*S. cerevisiae* TB39 $\rho^-$  ( $\alpha$ , pho9, his3, leu2), which is a respiratory-deficient mutant strain, is obtained by treating with ethidium bromide, one of strains obtained by hybridizing *S. cerevisiae* NA74-3A ( $\alpha$ , pho9, his4, leu2) (Japanese Patent Application No. 63-283716/1988 which corresponds to EP-317,209) with *S. cerevisiae* DK-13D ( $\alpha$ , leu2, trp1, his3) [*Molecular and Cellular Biology* 4, 771 (1984)].

The above hybridization is known per se, and conducted, for example, in accordance with the method described in *Experimental Methods of Microbiology*, edited by Microbiology Studying Method Conference, Kodansha, p.320 (1975).

The method for obtaining the respiratory-deficient strain ( $\rho^-$  strain) by treatment with ethidium bromide is known per se, and described, for example, in *Laboratory Course Manual for Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1986).

This *S. cerevisiae* TB39 $\rho^-$  strain is high in expression amount of genes, and therefore useful as a host for producing transformants.

The microorganisms produced in Reference Example and Examples 3 and 5 shown below were deposited in the Institute for Fermentation, Osaka, Japan (IFO), and in Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry Japan, (FRI) under the Budapest treaty. Their accession numbers and deposit dates are shown in Table 1.

Table 1

	Microorganism	IFO	FRI
5	<i>S. cerevisiae</i> TB39 $\rho^-$	IFO 10467 (April 24, 1989)	FERM BP-2399 (April 25, 1989)
	<i>S. cerevisiae</i> TB39 $\rho^-$ /pTB1011	IFO 10468 (April 24, 1989)	FERM BP-2400 (April 25, 1989)
	<i>S. cerevisiae</i> TB39 $\rho^-$ /pTB1012	IFO 10469 (April 24, 1989)	FERM BP-2401 (April 25, 1989)
10	<i>S. cerevisiae</i> TB39 $\rho^-$ /pTB1130	IFO 10500 (March 30, 1990)	FERM BP-2852 (April 7, 1990)
	<i>S. cerevisiae</i> TB39 $\rho^-$ /pTB1131	IFO 10501 (March 30, 1990)	FERM BP-2853 (April 7, 1990)
	<i>E. coli</i> DH1/pTB1163	IFO 15020 (March 30, 1990)	FERM BP-2854 (April 7, 1990)
	<i>E. coli</i> DH1/pTB1164	IFO 15021 (March 30, 1990)	FERM BP-2855 (April 7, 1990)
15	<i>E. coli</i> DH1/pTB1165	IFO 15022 (March 30, 1990)	FERM BP-2856 (April 7, 1990)
	<i>E. coli</i> DH1/pTB1166	IFO 15023 (March 30, 1990)	FERM BP-2857 (April 7, 1990)

20 The present invention will hereinafter be described in detail with the following Reference Example and Examples. It is understood of course that these are not intended to limit the scope of the invention.

#### 25 Reference Example Preparation of *S. cerevisiae* TB39 $\rho^-$

*S. cerevisiae* NA74-3A ( $\alpha$ , *pho9*, *his4*, *leu2*) was hybridized with *S. cerevisiae* DK-13D ( $\alpha$ , *leu2*, *trp1*, *his3*) [*Molecular and Cellular Biology* 4, 771 (1984)]. Then, one of the resulting strains was treated with ethidium bromide to obtain *S. cerevisiae* TB39 $\rho^-$  ( $\alpha$ , *MATa*, *leu2*, *his3*, *pho9*,  $\rho^-$ ) (IFO 10467, FERM BP-2399).

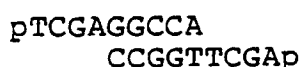
#### Example 1 Construction of Secretory Expression Vector for Yeast Having Promoter of $\alpha$ -Factor and Prepro Region

35 Plasmid p69A having a promoter of  $\alpha$ -factor and a prepro region [J. Kurjan and I. Herskowitz, *Cell* 30, 933-943 (1982)] was digested with restriction enzyme *EcoRI* to cut out a 1.6-kbp fragment having the promoter of  $\alpha$ -factor and the prepro region. This fragment was treated with *E. coli* polymerase Klenow fragments to change the termini thereof to flush ends. Then, a *BamHI* linker represented by p-(CCGGATCCGG) was ligated thereto with T4 DNA ligase, followed by cleavage with restriction enzymes

40 *BamHI* and *HindIII* to obtain a 0.9-kbp fragment.  
This DNA fragment was inserted into *BamHI* and *HindIII* sites of phage M13mp18 to prepare a single-stranded phage DNA (ssDNA). Using this ssDNA and a synthetic primer represented by 5'-TTTATCCAAGCTTACCCTTC-3', site-directed mutagenesis was carried out. This reaction was conducted by using a site-directed mutagenesis kit (Amersham, UK). A double-stranded DNA (RF-DNA) was prepared

45 from the resulting mutant phage, and digested with *BamHI* and *HindIII* to prepare a *BamHI*-*HindIII* fragment (0.9 kbp). This fragment was 25 bp shorter than the DNA fragment (0.9 kbp) first prepared.  
On the other hand, plasmid pGEL125 obtained by the method described in Yoshimura et al., *Biochem. Biophys. Res. Commun.* 145, 712 (1987) was digested with *HindIII*, followed by treatment with Klenow fragments. Then, cleaved portions were re-ligated with T4 DNA ligase to obtain pGEL125H. This was further

50 cleaved with *XhoI*, and a *XhoI*-*HindIII* adapter represented by



55 was introduced with T4 DNA ligase, followed by digestion with *HindIII* and *BamHI* to obtain a 8.3-kbp DNA fragment. This fragment (8.3 kbp) was ligated to the previously prepared fragment (0.9 kbp) with T4 DNA ligase to obtain pALFA310.

The XhoI-HindIII portion in the restriction enzyme map of the plasmid pALFA310T shown in Fig. 1 is indicated below in detail.

81

10 - - -Glu Gly Val Ser Leu - - - - -

- - -GAA GGG GTA ACC TTG GCCTCGAG - -

- - -CTT CCC CAT TCG AAC CGGAGCTC - -

15 Hind III Xho I

(The number 81 indicates the number in an amino acid sequence of  $\alpha$ -factor)

### Example 2 Introduction of Glycosylation Site into Human bFGF

### (1) Introduction into Amino Terminus

Site-directed mutagenesis was carried out using an ssDNA of phage clone M13PO into which a human bFGF cDNA was incorporated (European Patent Publication No. 281,822) and a synthetic primer represented by 5'-CTGCCGTATCCTCGGGCAAT-3'. A site directed mutagenesis kit (Amersham, UK) was used for this reaction. An RF DNA was prepared from the resulting mutant phage M13P14, and digested with 30 Aval and Sall to prepare a cDNA fragment lacking a cDNA portion corresponding to the 5 residues from the N-terminus of bFGF. Then, this fragment was inserted into the HindIII-XhoI site of pALFA310T, using a synthetic adapter (HindIII-Aval) represented by the following formula, to obtain pTB1011 (refer to Fig. 2).

35 5'-AGCTTGGATAAAAGACCAGCATTC-3'  
3' -ACCTATTTTCTGGTCGTAACGGGGCT- 5'

As a result, G<sup>7</sup> I<sup>7</sup> y of hbFGF was changed to Asn, and thereby a glycosylation site A<sup>7</sup> s n-G<sup>8</sup> I<sup>8</sup> y-S<sup>9</sup> e r<sup>9</sup> was formed in hbFGF (refer to Fig. 3), and the mutein is named as hbFGF mutein Ng.

## (2) Introduction into Carboxyl Terminus

45 Using an ssDNA of phage M13PO and a synthetic primer represented by 5'-ATCAACTCTTATTAGACATTG-3', site-directed mutagenesis was carried out as with (1). An RF DNA was prepared from the resulting mutant phage M13P15, and cleaved with *Ava*I and *Sal*I to prepare a cDNA fragment of hbFGF. Then, this fragment was inserted into the *Hind*III-*Xho*I site of pALFA310T, using the above synthetic adapter, to obtain pTB1012 (refer to Fig. 4).

50 As a result, <sup>14</sup>Ala of the hbFGF was changed to Asn, and thereby a glycosylation site

7 8 9  
Asn-Gly-Ser

<sup>55</sup> was formed in the hbFGF (refer to Fig. 5), and the mutein is named as hbFGF mutein Cg.

### Example 3 Production with Yeast of hbFGF Having Glycosylation Site

Using DNAs of plasmids pTB1011 and pTB1012, yeast *S. cerevisiae* TB39<sup>-</sup> was transformed with the method described in *Proc. Natl. Acad. Sci. USA*, 75, 1929(1978) to obtain transformants *S. cerevisiae* TB39<sup>-</sup>/pTB1011 (IFO 10468, FERM BP-2400) and *S. cerevisiae* TB39<sup>-</sup>/pTB1012 (IFO 10469, FERM BP-2401), respectively.

Each of the transformant strains thus obtained was cultivated in modified Burkholder medium containing 8.9% of sucrose and 1.1% of glucose [Toh-e et al., *J. Bacteriol.* 113, 727 (1973)] for 3 days. After cultivation, cells were isolated from the medium by centrifugation (3300 rpm, 15 minutes).

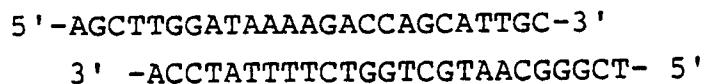
The cells collected from a culture volume of 1 ml were suspended in 100μl of a zymolase solution [200 μg/ml zymolase, 12 M sorbitol, 50 mM K-phosphate (pH 7.4), 0.1% mercaptoethanol] and allowed to stand at room temperature for 2 hours. Then, 400 μl of a triton X-100 solution [0.1% Triton X-100, 50 mM K-phosphate (pH 7.4), 1 mM PMSF] was added thereto, and the mixture was further allowed to stand at room temperature for 2 hours. Thereafter, 20-second ultrasonication was carried out twice under ice cooling, and a supernatant was collected by centrifugation (16,000 rpm, 30 minutes) as a crude extracted solution.

The bFGF activity of the resulting media and crude extracted solutions was assayed according to the amount of [<sup>3</sup>H] thymidine taken in BALB/c3T3 cells. The bFGF activity as shown in the following table was observed.

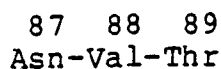
Transformant	Activity in Medium	Activity in Crude Extracted Solution
<i>S. cerevisiae</i> TB39 <sup>-</sup> /pTB1011	616	656
<i>S. cerevisiae</i> TB39 <sup>-</sup> /pTB1012	37	131
(Unit: ng of bFGF equivalent/ml of culture)		

#### Example 4 Introduction of Glycosylation Site into Human bFGF and hbFGF mutein CS2

Site-directed mutagenesis was carried out using an ssDNA of phage clone M13PO into which a human bFGF cDNA was incorporated (European Patent Publication No. 281,822) and an ssDNA of phage clone M13P2 into which a mutein CS2 cDNA was incorporated (European Patent Publication No. 281,822) and a synthetic primer represented by 5'-GTAACATTCTTAGAAGCCAGT-3'. A site directed mutagenesis kit (Amersham, UK) was used for this reaction. RF DNAs were prepared from the resulting mutant phages M13CN3 and M13CS2CN3 respectively, and digested with Aval and salI to prepare cDNA fragments lacking a cDNA portion corresponding to the 5 residues from the N-terminus of bFGF. Then, each of these fragments was inserted into the HindIII-XhoI site of pALFA310T, using a synthetic adapter (HindIII-Aval) represented by the following formula, to obtain pTB1130 and pTB1131 (refer to Figs. 6 and 7).



As a result, Cys<sup>87</sup> of bFGF and hbFGF mutein CS2 was changed to Asn, and thereby a glycosylation site



was formed in hbFGF and hbFGF mutein CS2 (refer to Figs. 8 and 9, and these muteins are named hbFGF mutein CN3 and hbFGF mutein CS2CN3 respectively).

#### Example 5 Production with Yeast of bFGF Having Glycosylation Site

Using DNAs of plasmids pTB1130 and pTB1131, yeast *S. cerevisiae* TB39<sup>-</sup> was transformed to obtain

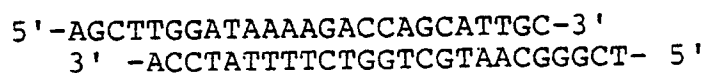
Each of the transformants thus obtained was cultivated in modified Burkholder medium containing 8.9% of sucrose and 1.1% of glucose [Toh-e et al., J. Bacteriol. **113**, 727 (1973)] for 48 hours. After cultivation, cells were removed from the medium by centrifugation (3300 rpm, 15 minutes).

The bFGF activity of the resulting media was assayed according to the amount of [<sup>3</sup>H] thymidine incorporated in BALB/c3T3 cells. The bFGF activity as shown in the following table was observed.

Transformant	48 hours
<u>S. cerevisiae</u> TB39 $\rho^-$ /pTB1130	725
<u>S. cerevisiae</u> TB39 $\rho^-$ /pTB1131	816
(Unit: ng of bFGF equivalent/ml of culture)	

### Example 6 Introduction of Glycosylation Site into Human bFGF

Site-directed mutagenesis was carried out using an ssDNA of phage clone M13CN3 into which a human bFGF cDNA was incorporated and a synthetic primer represented by 5'-GTAACGGGTAGCATTCACTCCTT-3'. A site directed mutagenesis kit (Amersham, UK) was used for this reaction. An RF DNA was prepared from the resulting mutant phage M13CN23, and digested with *Ava*I and *Sal*I to prepare a cDNA fragment lacking a cDNA portion corresponding to the 5 residues from the N-terminus of bFGF. Then, this fragment was inserted into the *Hind*III-*Xho*I site of pALFA310T, using a synthetic adapter (*Hind*III-*Ava*I) represented by the following formula, to obtain an expression plasmid pTB1172(refer to Fig. 10).



35 As a result,

69 71 87 69  
Cys, Asn, Cys of bFGF were changed to Asn,

40                    71        87  
                  Thr, Asn, respectively and thereby glycosylation sites

69 70 71 87 88 89  
Asn-Ala-Thr and Asn-Val-Thr

<sup>45</sup> were formed in bFGF (refer to Fig. 11, and this mutein is named as hbFGF mutein CN23.

Using DNAs of plasmids thus obtained, yeast *S. cerevisiae* TB39 $\rho^-$  is transformed to obtain transformants.

Each of the transformants thus obtained is cultivated in modified Burkholder medium containing 8.9% of sucrose and 1.1% of glucose [Toh-e et al., J. Bacteriol. **113**, 727 (1973)]. After cultivation, cells are removed from the medium by centrifugation (3300 rpm, 15 minutes).

The bFGF activity of the resulting media is assayed according to the amount of [ $^3\text{H}$ ] thymidine incorporated in BALB/c3T3 cells.

55 Example 7 Purification of CN<sub>3</sub>

500 ml of a culture supernatant of *S. cerevisiae* TB39  $\rho^-$ /pTB1130 cultivated at 28 °C for 48 hours was

allowed to pass through a chelating Sepharose column (2.5 cm in diameter X 7 cm in length), and then, applied to a heparin-copper bioaffinity column [Y. Shing, J. Biol. Chem. 263, 9059-9062 (1988)]. 90 ml of 1 M NaCl-20 mM Tris-HCl (pH 7.4), 40 ml of 0.2 M NaCl-20 mM Tris-HCl (pH 7.4) and 100 ml of 10 mM imidazole-0.2 M NaCl-20 mM Tris-HCl (pH 7.4) were allowed to flow through the column in order, and the effluent was fractionated for every 5 ml from the time that the first 30 ml thereof flowed. Then, 10 mM imidazole-2 M NaCl-20 mM Tris-HCl (pH 7.4) was allowed to flow through the column, and the effluent was fractionated for every 2.5 ml. 10  $\mu$ l of each of the resulting fractions was assayed by the 2-site ELISA using heparin as a primary ligand and anti-bFGF antibody as a secondary ligand [Y. Sato et al., Mol. Endocrinol. 3, 744-748 (1989)]. As a result, the immunoreactivity was observed at an effluent volume of about 250 ml to 280 ml. The fractions at effluent volumes of 260 ml to 272.5 ml (fraction Nos. 65 to 69) were collected, and examined by SDS-PAGE. Consequently, three stained bands were observed in the region corresponding to molecular weights of 21 to 23 kDa (Fig. 12A). In a Western blotting method using an anti-FGF antibody, three bands were also observed at the positions of 21 to 23 kDa. These bands shifted to the electrophoresis position of bFGF (16.5 kDa) and the position of 17.4 kDa by EndoH treatment (Fig. 12B). With respect to three kinds of proteins having a molecular weight of 20 to 23 kDa, it was considered from these results that a sugar chain was ligated to bFGF and to bFGF added an amino acid sequence derived from an  $\alpha$ -factor signal peptide. The biological activity of the purified sample (fraction Nos. 65 to 69) was examined by introduction of DNA synthesis in BALB/c3T3 cells. As a result, the activity was 7.8  $\mu$ g FGF equivalent/ml, and the protein concentration was 10.8  $\mu$ g/ml, which revealed that its specific activity was 72% of bFGF (Table 2). The total activity of the purified sample was 97.5  $\mu$ g FGF equivalent, and the recovery was 29.8% (Table 2).

Table 2

	Volume (ml)	FGF Activity (ng/ml)	Total Activity ( $\mu$ g)	Recovery (%)
<i>S. cerevisiae</i> TB39 <sup>p</sup> /pTB1130 culture supernatant	500	647	323.5	100
Peak fraction	12.5	7800	97.5	29.8

#### Example 8 Purification of CS2/CN3

400 ml of a culture supernatant of *S. cerevisiae* TB39<sup>p</sup> /pTB1131 cultivated at 28°C for 48 hours was allowed to pass through a chelating Sepharose column (1.4 cm in diameter X 10 cm in length), and then, applied to a heparin-copper bioaffinity column [Y. Shing, J. Biol. Chem. 263, 9059-9062 (1988)]. 140 ml of 1 M NaCl-20 mM Tris-HCl (pH 7.4), 110 ml of 0.2 M NaCl-20 mM Tris-HCl (pH 7.4) and 200 ml of 10 mM imidazole-0.2 M NaCl-20 mM Tris-HCl (pH 7.4) were allowed to flow through the column in order, and the effluent was fractionated for every 5 ml. Then, 10 mM imidazole-2 M NaCl-20 mM Tris-HCl (pH 7.4) was allowed to flow through the column, and the effluent was fractionated for every 2 ml. 10  $\mu$ l of each of the resulting fractions was assayed by the 2-site ELISA using heparin as a primary ligand and anti-bFGF antibody as a secondary ligand [Y. Sato et al., Mol. Endocrinol. 3, 744-748 (1989)]. As a result, the strong immunoreactivity was observed at an effluent volume exceeding about 500 ml. The fractions at effluent volumes of 504 ml to 518 ml (fraction Nos. 118 to 124) were collected as a peak fraction. The peak fraction was assayed by SDS-PAGE. Consequently, stained bands corresponding to molecular weights of 21 to 23 kDa characteristic of sugar chain addition type bFGF were observed. In a Western blotting method, bands were also observed at the same positions (Fig. 15). The total FGF activity of the peak fraction was 84.8  $\mu$ g FGF equivalent, and the recovery was 49% (Table 3).

Table 3

	Volume (ml)	FGF Activity (ng/ml)	Total Activity ( $\mu$ g)	Recovery (%)
<u>S. cerevisiae</u> TB39 $\rho^-$ /pTB1131 culture supernatant	400	427	170.8	100
Peak fraction	14	6060	84.8	49

#### Example 9 Expression of Gene Coding for Human bFGF Mutein (Sugar Chain Addition Type) in Animal Cells

##### (1) Construction of Plasmid pTB1141 for Expression of Human bFGF Mutein (Sugar Chain Addition Type)

An RF-type DNA of phage M13 CN3 obtained in the above Example 4 was cleaved with restriction enzyme EcoRI-BamHI to obtain a 0.38-kb DNA fragment containing a region coding for human bFGF mutein CN3. Further, plasmid pTB503 described in European Patent Application Publication No. 225,701 was cleaved with ClaI-EcoRI to obtain a 1.7-kb DNA fragment containing a murine leukemia virus (MuLV) LTR region, an SV40-derived promoter and splice junction, and a human interleukin 2 (IL-2) leader sequence. In addition, plasmid pTB675 described in European Patent Application Publication No. 281,822 was cleaved with ClaI-BamHI to obtain a 3.4-kb DNA fragment containing a coding region on the C-terminal side of human bFGF, and a 3'-untranslated region, a plasmid pBR322-derived ampicillin-resistant gene and a replication origin in Escherichia coli. These three kinds of DNA fragments were ligated to one another using T4DNA ligase to obtain plasmid pTB1141 (Fig. 14).

##### (2) Expression in Animal Cells

Monkey COS-7 cells were seeded in DMEM medium containing 10% fetal calf serum in a 6 cm-diameter dish for tissue culture, and the medium was changed for the same medium the next day. After 4 hours, 10  $\mu$ g of the plasmid pTB1141 DNA was transfected by the calcium phosphate method [Graham et al., Virology 52, 456 (1973)]. The next day, the medium was changed for DMEM medium containing 0.5% fetal calf serum, and cultivation was continued. After 48 hours, the culture medium and the cells were collected. The cells were washed with PBS twice, and then suspended in 1 M NaCl-20 mM Tris-HCl (pH 7.6). After ultrasonication for a short time, the suspension was centrifuged at 15,000 rpm for 15 minutes to obtain a supernatant. For the culture medium of the plasmid-infected COS-7 cells and the cell extract, the FGF activity was measured by the method described in Mol. Cell. Biol. 8, 588(1988). The results are shown in Table 4.

Table 4

Plasmid	FGF Activity (ng/dish)	
	Culture Medium	Cell Extract
pTB1141	530	491
---	< 0.02	1

For 0.5 ml of the culture solution, the product was further analyzed by a Western blotting method. As a result, the bands of 16 kDa and about 20 kDa which were the same as with rhbFGF were detected from the culture supernatant of the COS-7 cells transfected with pTB1141. The band of 20 kDa became not to be



detected in the culture supernatant of the pTB1141-transfected COS-7 cells in the presence of tunicamycin. From this fact, the production of sugar chain addition type bFGF was confirmed.

When mouse BALB/c3T3 A31 cells were transfected with pTB1141, focus formation was observed, and the resulting transformed cells exhibited colony forming ability in soft agar medium and tumorigenesis in nude mice.

(3) Construction of Plasmids pTB1163, 1164, 1165 and 1166 for Expression of Human bFGF Mutein (Sugar Chain Addition Type) (Refer to the construction of the plasmid 1162 in Fig. 15)

Plasmid pTB675 described in European Patent Application Publication No. 281,822 was cleaved with restriction enzyme NcoI, and an attached terminus was changed to a flush end by a DNA polymerase Klenow fragment reaction, followed by cleavage with ClaI to obtain a 3.8-kb DNA fragment containing a human bFGF structural gene, a 3'-untranslated region, an SV40-derived poly(A) addition region, a pBR322-derived ampicillin-resistant gene and a replication origin. Further, the plasmid pTB503 described in the above item (1) was cleaved with ClaI-HindIII to obtain a 1.1-kb DNA fragment containing a MuLV LTR region. In addition, plasmid pTB106 [Cell Struct. Funct. 12 205 (1987)] was cleaved with HgiAI to isolate and purify a 1.0-kb DNA fragment containing an SV40 promoter region and a human IL-2 cDNA leader sequence. The termini were changed to flush ends by a T4 polymerase reaction, followed by cleavage with HindIII to obtain a 0.64-kb DNA fragment. These three kinds of DNA fragments were ligated to one another to obtain plasmid pTB1001. The plasmid pTB1001 have a structure so as to express the human bFGF structural gene (-9 to 146 amino acids) ligated to the IL-2 leader sequence in a same reading frame under the control of the MuLV LTR and the SV40 early promoter.

Then, a 1.3-kb DNA fragment which was obtained by cleaving pTB1001 with HindIII and contained a human bFGF gene ligated to a human IL-2 leader sequence was cloned to the HindIII site of plasmid pUC119 (Takara Shuzo, Japan).

Using synthetic oligonucleotide 5'-GTAACATTCTTAGAAGCCAGT as a primer and an in vitro mutagenesis system version 2 kit (Amersham), site-directed mutagenesis was carried out according to a specified method to obtain a CN3 mutant IL-CN3-155 (plasmid pTB1158) which comprises IL-2 leader sequence and hbFGF mutant CN3 sequence of -9 to 146. Further, using synthetic oligonucleotide CS2 as a primer, a CS2/CN3 mutant IL-CS2/CN3-155 (plasmid pTB1160), which comprises IL-2 leader sequence and hbFGF mutein CS2CN3 sequence of -9 to 146, was obtained by a similar method.

Using synthetic oligonucleotide 31-mer 5'CTCGGGCAAGGCGGGACTGTTTGTGACAAGT<sup>3</sup>, human bFGF amino acid

Met    Leu  
Nos.-9 to -1

was removed, and an IL-2 leader sequence and a mature human bFGF [amino acid Nos. 1(Pro) to 146] were ligated thereto by using the above kit to prepare IL-F146 (plasmid pTB1155) which comprises IL-2 leader sequence and hbFGF sequence of 1 to 146. The above synthetic oligonucleotides for CN3 mutation and CS2/CN3 mutation were applied thereto to obtain mutants IL-CN3-146 (plasmid pTB1159) (which comprises IL-2 leader sequence and hbFGF mutein CN3 sequence of 1 to 146) and IL-CS2/CN3-146 (plasmid pTB1161) (which comprises IL-2 leader sequence and hbFGF mutein CS2/CN3 sequence of 1 to 146), respectively.

Each of pUC119 plasmids containing the mutant genes thus obtained was cleaved with NcoI-HindIII, and each of 1.1-kb DNA fragments containing the mutant genes was isolated. Each of the isolated DNA fragments, the 3.1-kb ClaI-HindIII DNA fragment obtained from the above expression plasmid pTB1001 for animal cells and a 1.4-kb ClaI-NcoI DNA fragment were ligated to one another by a T4 DNA ligase reaction to construct the following plasmids:

pTB1162: IL-F146  
pTB1163: IL-CN3-146  
pTB1164: IL-CS2/CN3-146  
pTB1165: IL-CN3-155  
pTB1166: IL-CS2/CN3-155

Using plasmid pTB1163, pTB1164, pTB1165 or pTB1166, *E. coli* DH1 was transformed with the method described in Molecular Cloning, Cold Spring Harbor Laboratories, page 249, 1982 to obtain transformants *E. coli* DH1/pTB1163 (IFO 15020, FERM BP-2854), *E. coli* DH1/pTB1164(IFO 15021, FERM BP-2855), *E. coli*

DH1/pTB1165 (IFO 15022, FERM BP-2856) and E. coli DH1/pTB1166 (IFO 15023, FERM BP-2857), respectively.

#### 5 (4) Expression in Animal Cells

Monkey COS-7 cells are transfected with pTB1163, pTB1164, pTB1165 and pTB1166 DNAs by the method described in item (2) of this example, and thereby sugar chain addition type human bFGFs can be obtained from their cell culture. When Mouse BALB/c3T3 A31 cells are transfected with them, transformed  
10 cells can be obtained.

#### (5) Construction of plasmids pTB1178 and pTB1179 for Expression of Human bFGF Mutein (Sugar Chain Addition Type)

15

Site-directed mutagenesis is carried out using a synthetic primer represented by 5'-GTAACGGGTAGCATTCACTCCTT-3' and M13phage ssDNAs prepared from E. coli MV1184 having plasmid pTB1158 and pTB1159, respectively. Site-directed mutagenesis kit (Amersham, UK) is used for this reaction. The RF DNAs are prepared from the resulting mutant phages and digested with NcoI-HindIII to  
20 obtain the 1.1 kb of DNA fragments. Then, using these two fragments, two plasmids, pTB1178 (IL-CN23-146) and pTB1179 (IL-CN23-155) for expression of human bFGF muteins are constructed by the procedure described in said items (1) and (3).

#### 25 (6) Expression in Animal Cells

Monkey COS-7 cells are seeded in DMEM medium containing 10% fetal calf serum in a 6 cm-diameter dish for tissue culture, and the medium is changed for the same medium the next day. After 4 hours, 10 µg of the plasmid pTB1178 DNA or pTB1179 DNA is transfected by the calcium phosphate method [Graham et al., Virology 52, 456 (1973)]. The next day, the medium is changed for DMEM medium containing 0.5% fetal calf serum, and cultivation is continued. After 48 hours, the culture medium and the cells are collected. The cells are washed with PBS twice, and then suspended in 1 M NaCl-20 mM Tris-HCl (pH 7.6). After ultrasonication for a short time, the suspension is centrifuged at 15,000 rpm for 15 minutes to obtain a supernatant. For the culture medium of the plasmid-infected COS-7 cells and the cell extract, the FGF  
35 activity is measured by the method described in Mol. Cell. Biol. 8, 588(1988).

### Example 10 Stability of Sugar Chain Addition Type bFGF

40

#### (1) Stability of hbFGF mutein CN3 Produced by Yeast

The stability of the muteins in medium 8S5N was assayed at 37° C after 0 to 20 hours.

Each of hbFGF mutein CN3 and hbFGF mutein CS23 was adjusted to a specific activity of 500 ng/ml, and incubated at 37° C. The specific activity of each sample at 0 hour was taken as 100%, and the activity  
45 at the time that sampling was carried out was expressed in % (Fig. 16).

As a result, hbFGF mutein CN3 showed a tendency more stable than hbFGF mutein CS23, and stabilization due to sugar addition was suggested.

50

#### (2) Stability of hbFGF mutein CN3 Produced in Animal Cells

The stability of the muteins in medium DMEM was assayed at 37° C after 0 to 20 hours.

Using expression systems in which IL-2 signal peptides were ligated to the N-termini of hbFGF (pTB1085), hbFGF mutein CS23 (pTB831) (EP-281,822) and hbFGF mutein CN3(pTB1141), respectively,  
55 production was carried out in COS-7 cells, and each of culture supernatants was incubated at 37° C.

The specific activity of each sample at 0 hour was taken as 100%, and the residual activity at each time was expressed in % (Fig. 17).

As a result, it was suggested that hbFGF mutein CN3 was excellent in stability.

The above-described plasmid pTB1085 was obtained by inserting MuLVLTR DNA sequence of pTB504 into ClaI and HindIII sites of pTB675 (EP-281,822), removing 5'-untranslated region of hbFGF cDNA from the pTB675 with PstI and NcoI and ligating the sites.

5

## Claims

1. A mutein of a fibroblast growth factor (FGF) into which has been introduced at least one glycosylation site.
2. The mutein according to claim 1, which has one or more sugar chains.
3. The mutein according to claim 2, wherein the sugar is selected from at least one of the group consisting of N-acetyl glycosamine, N-acetyl galactosamine, mannose, galactose, fucose and cyalic acid.
4. The mutein according to claim 1, wherein the glycosylation site comprises an amino acid sequence represented by the following formula:  
Asn-X-Y  
wherein X is any amino acid residue, and Y is Thr, Ser or Cys.
5. The mutein according to claim 4, wherein X is Gly, Lys, Val or Ala.
6. The mutein according to claim 4, wherein Y is Thr or Ser.
7. The mutein according to claim 1, wherein the FGF comprises a human basic FGF.
8. A DNA coding for a mutein of a fibroblast growth factor (FGF), the DNA having introduced therein at least one nucleotide sequence coding for a glycosylation site.
9. A vector containing a DNA coding for a mutein of a fibroblast growth factor (FGF), the DNA having introduced therein at least one nucleotide sequence coding for a glycosylation site.
10. A transformant transformed with a vector containing a DNA coding for a mutein of a fibroblast growth factor (FGF), the DNA having introduced at least one nucleotide sequence coding for a glycosylation site.
11. The transformant according to claim 10, whose host is yeast or animal cell.
12. A process for producing a mutein of a fibroblast growth factor (FGF), into which has been introduced at least one glycosylation site which comprises;  
cultivating in a culture medium a yeast or animal cell transformant transformed with a vector containing a DNA coding for a mutein of a fibroblast growth factor (FGF), the DNA having introduced therein at least one nucleotide sequence coding for a glycosylation site, and producing and accumulating said mutein in the culture medium.

35

Claims for the following Contracting States: ES and GR

1. A process for producing a mutein of a fibroblast growth factor (FGF), into which has been introduced at least one glycosylation site which comprises;  
cultivating in a culture medium a yeast or animal cell transformant transformed with a vector containing a DNA coding for a mutein of a fibroblast growth factor (FGF), the DNA having introduced therein at least one nucleotide sequence coding for a glycosylation site, and producing and accumulating said mutein in the culture medium.
2. The process according to claim 1, wherein the mutein has one or more sugar chains.
3. The process according to claim 2, wherein the sugar is selected from at least one of the group consisting of N-acetyl glycosamine, N-acetyl galactosamine, mannose, galactose, fucose and cyalic acid.
4. The process according to claim 1, wherein the glycosylation site comprises an amino acid sequence represented by the following formula:  
Asn-X-Y  
wherein X is any amino acid residue, and Y is Thr, Ser or Cys.
5. The process according to claim 4, wherein X is Gly, Lys, Val or Ala.
6. The process according to claim 4, wherein Y is Thr or Ser.
7. The process according to claim 1, wherein the FGF comprises a human basic FGF.

55

Fig. 1 -1

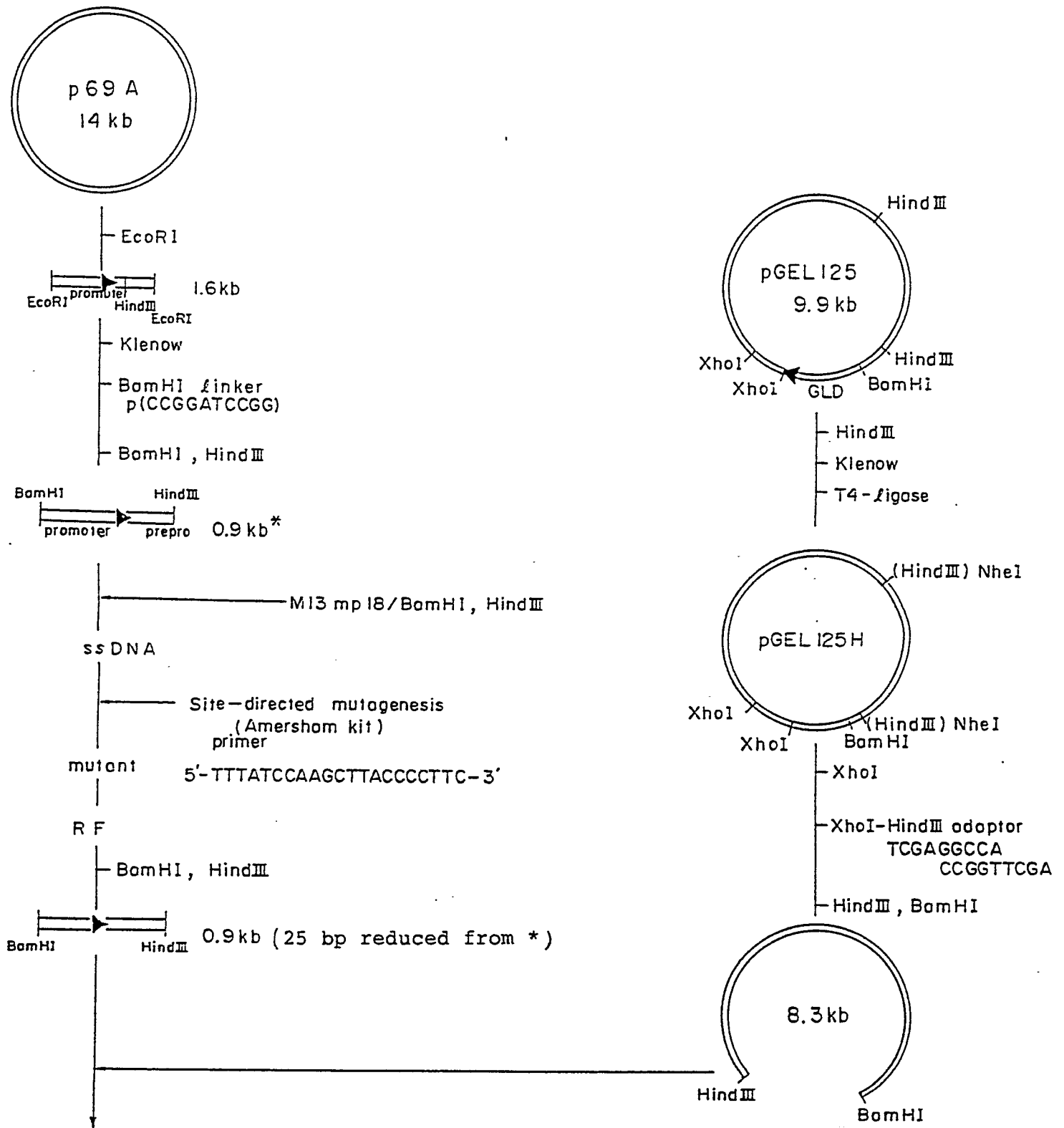


Fig. 1 -2

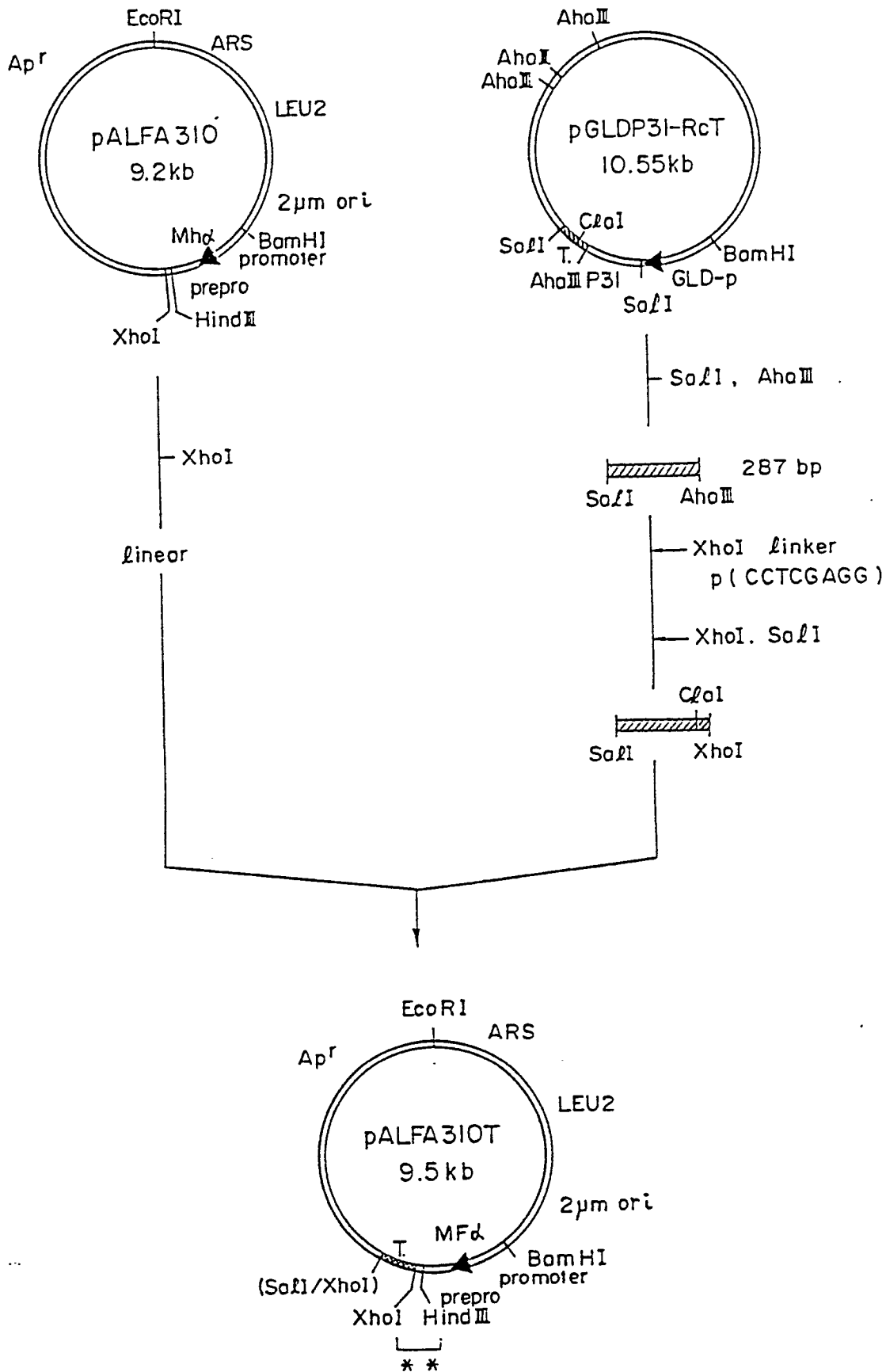


Fig. 2

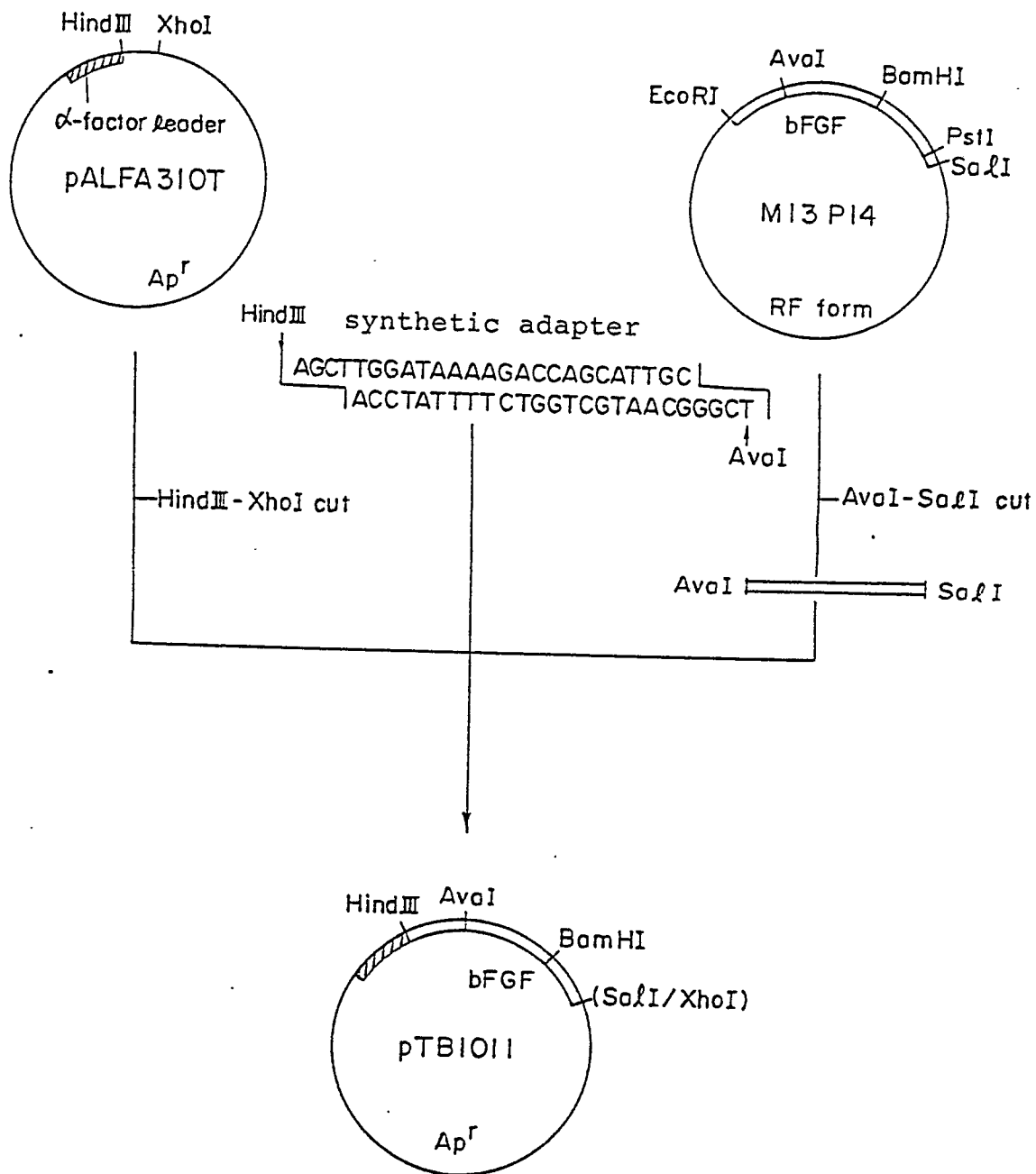


Fig. 3

$\alpha$ -factor  $\leftarrow$   
 SerLeuAspLysArg  
 (A) AGCTTGGATAAAAGA\*  
 HindIII

/  
 \*ProAlaLeuProGluAspAsnGlySerGlyAlaPheProProGlyHisPheLysAsp  
 CCAGCATTGCCGAGGATAACGGCAGGGCGCCTTCCCGCCGCCACTTCAAGGAC

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg  
 CCCAAGCGGCTGTACTGCAAAACGGGGCTTCTTCCCTGCGCATCCACCCGACGCCGA

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuAlaGluGlu  
 GTTGACGGGTCCGGGAGAGAGCGACCCCTCACATCAAGCTACAACCTTCAAGCAGAAGAG

ArgGlyValValSerIleLysGlyValCysAlaAsnArgTyrLeuAlaMetLysGluAsp  
 AGAGGAGTTGTGTCTATCAAAAGGAGTGTGTGCTAACCCGTTACCTGGCTATGAAGGAAGAT

GlyArgLeuLeuAlaSerLysCysValThrAspGluCysPhePheGluArgLeuGlu  
 GGAAGATTACTGGCTTCTAAATGTGTACGGATGAGTGTTCTTTTGAACGATTGGAA

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys  
 TCTAATAACTACAATACTTACCCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe  
 CGAACTGGGCAGTATAAACTTGGATCCAAACAGGACCTGGGCAGAAAGCTATACTTTT

/46  
 LeuProMetSerAlaLysSerTrm  
 CTTCCAATGTCTGCTAAGAGCTGA

Fig. 4

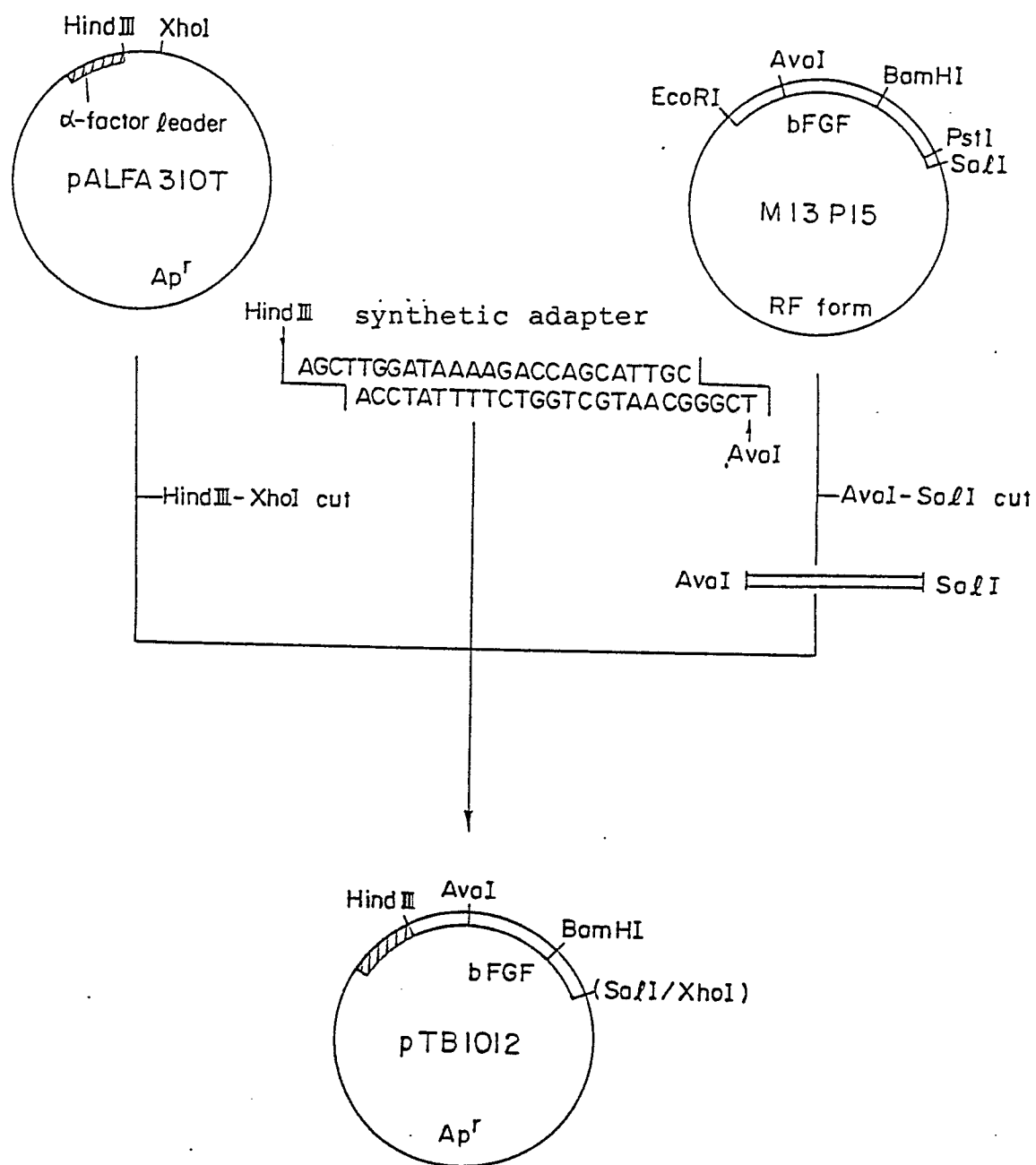




Fig. 5

$\alpha$ -factor ←  
 SerLeuAspLysArg  
 (A) AGCTTGGATAAAAGA\*  
 HindIII

\*ProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp  
 CCAGCATTGCCCGAGGATGGCGGCAGCGGCCCTTCCCGCCGCCACTTCAAGGAC

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg  
 CCCAAGCGGCTGTA CTGCAAAACGGGGGCTTCTTCTGGCATCCACCCGACGGCCGA

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu  
 GTTGACGGGGTCCGGGAGAGAGCGACCCCTCACATCAAGCTACAACTTCAAGCAGAAAGAG

ArgGlyValValSerIleLysGlyValCysAlaAsnArgTyrLeuAlaMetLysGluAsp  
 AGAGGAGTTGTGCTATCAAAAGGAGTGTGTGCTAACCGTTACCTGGCTATGAAGGAAGAT

GlyArgLeuLeuAlaSerLysCysValThrAspGluCysPhePheGluArgLeuGlu  
 GGAAGATTACTGGCTTCTAAATGTGTACGGATGAGTGTTCTTTTGAACGATTGGAA

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys  
 TCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe  
 CGAACTGGGCAGTATAAACTTGGATCCAAACAGGACCTGGGCAGAAAGCTACTTTT

LeuProMetSerAsnLysSert<sup>186</sup>rm  
 CTCCAATGTCTAATAAGAGTTGA

Fig. 6

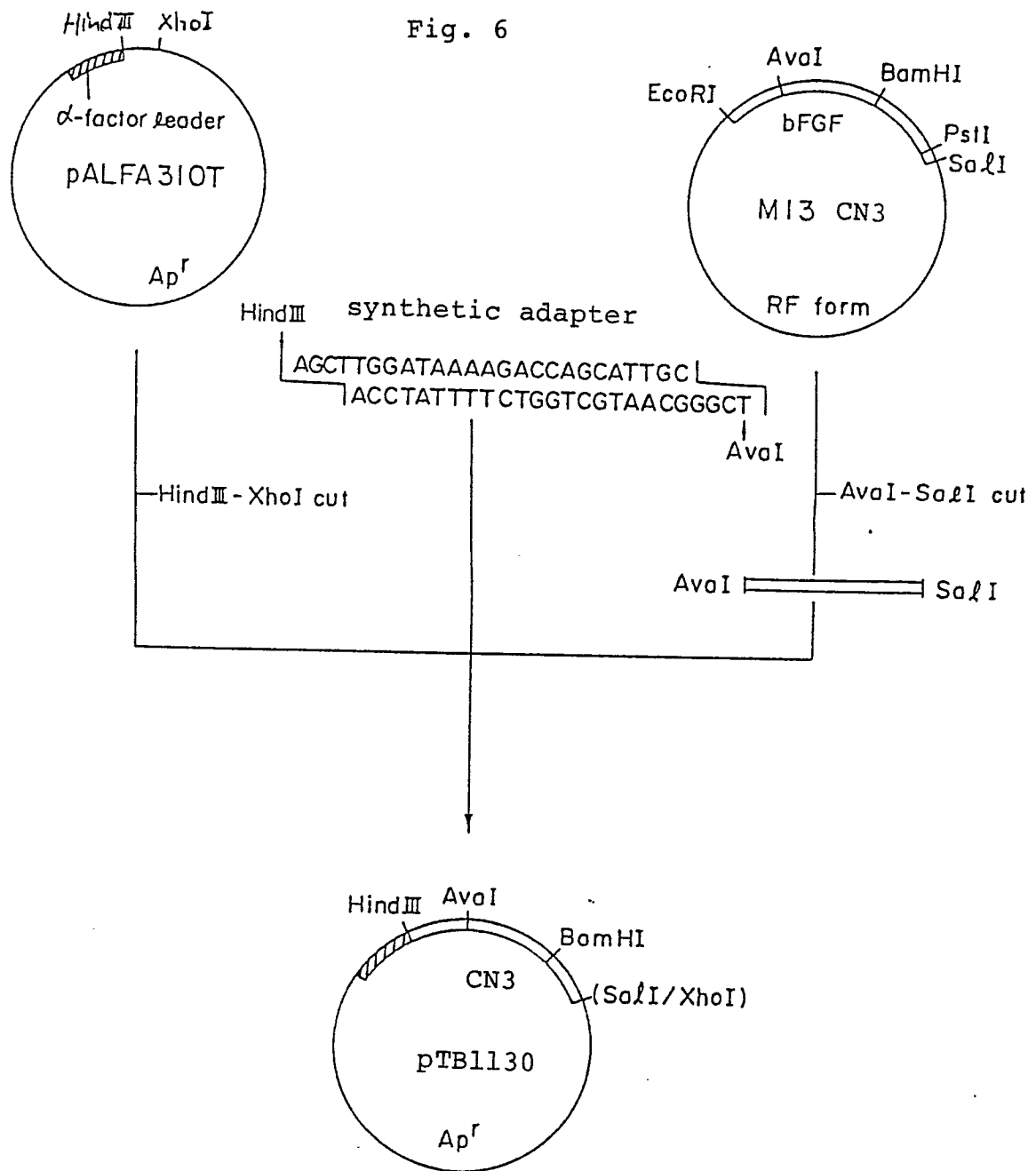


Fig. 7

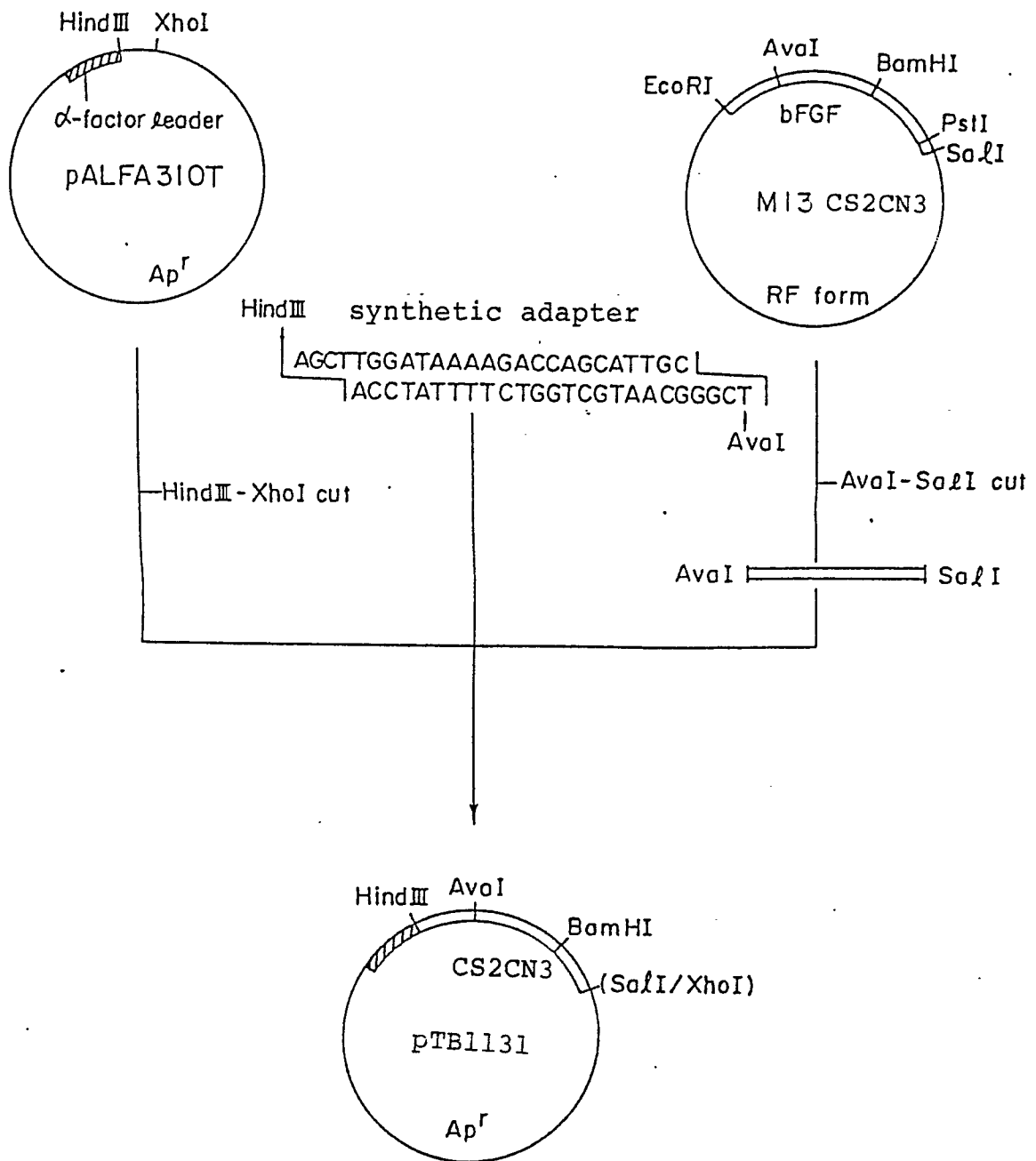


Fig. 8

$\alpha$ -factor  $\leftarrow$   
 SerLeuAspLysArg  
 (A) AGCTTGGATATAAAGA\*  
 .HindIII

/  
 \*ProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp  
 CCAGCATTTGCCCGAGGATGGCGGCAGCGGCCCTTCCCCCGCCCACTTCAAGGAC

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg  
 CCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCTGGCATCCACCCGACGGCCGA

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu  
 GTTGACGGGTCCGGAGAGAGCGACCCCTCACATCAAGCTACAACACTTCAAGCAGAAGAG

ArgGlyValValSerIleLysGlyValCysAlaAsnArgTyrLeuAlaMetLysGluAsp  
 AGAGGAGTTGTGTCTATCAAAGGAGTGTGTGCTAACCGTTACCTGGCTATGAAGGAAGAT

GlyArgLeuLeuAlaSerLysAsnValThrAspGluCysPhePheGluArgLeuGlu  
 GGAAAGATTACTGGCTTCTAAGAAATGTTACGGATGAGTGTTCTTTTGAACGATTGGAA

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys  
 TCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe  
 CGAACTGGGCAGTATAAACTTGGATCCAAAACAGGACCTGGGCAGAAAGCTATACTTTT

/46  
 LeuProMetSerAlaLysSerTrm  
 CTTCCAAATGCTGCTAAGAGCTGA

Fig. 9

$\alpha$ -factor ←  
 SerLeuAspLysArg  
 (A) AGCTTGGATAAAAGA\*  
 HindIII

/  
 \*ProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp  
 CCAGCATTGCCCGAGGATGGCGGCAGCGGCCCTTCCCGCCGCACTTCAAGGAC

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg  
 CCCAAGCGGCTGTACTGCAAAAACGGGGGCTTCTTCCTGCGCATCCACCCGACGGCCGA

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu  
 GTTGACGGGTCCGGAGAGAGCGACCCCTCACATCAAGCTACAACTTCAAGCAGAAAGAG

ArgGlyValValSerIleLysGlyValSerAlaAsnArgTyrLeuAlaMetLysGluAsp  
 AGAGGAGTTGTGTCTATCAAAAGGAGTGAGCGCTAATCGTTACCTGGCTATGAAGGAAGAT

GlyArgLeuLeuAlaSerLysAsnValThrAspGluCysPhePheGluArgLeuGlu  
 GGAAGATTACTGGCTTCTAAGAAATGTTACGGATGAGTGTTCTTTTGAACGATTGGAA

SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys  
 TCTAAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA

ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe  
 CGAACTGGGCAGTATAAACTTGGATCCAAACAGGACCTGGGCAGAAAGCTATACTTTT

/46  
 LeuProMetSerAlaLysSertrm  
 CTTCCAATGTCTAAGAGCTGA

Fig. 10

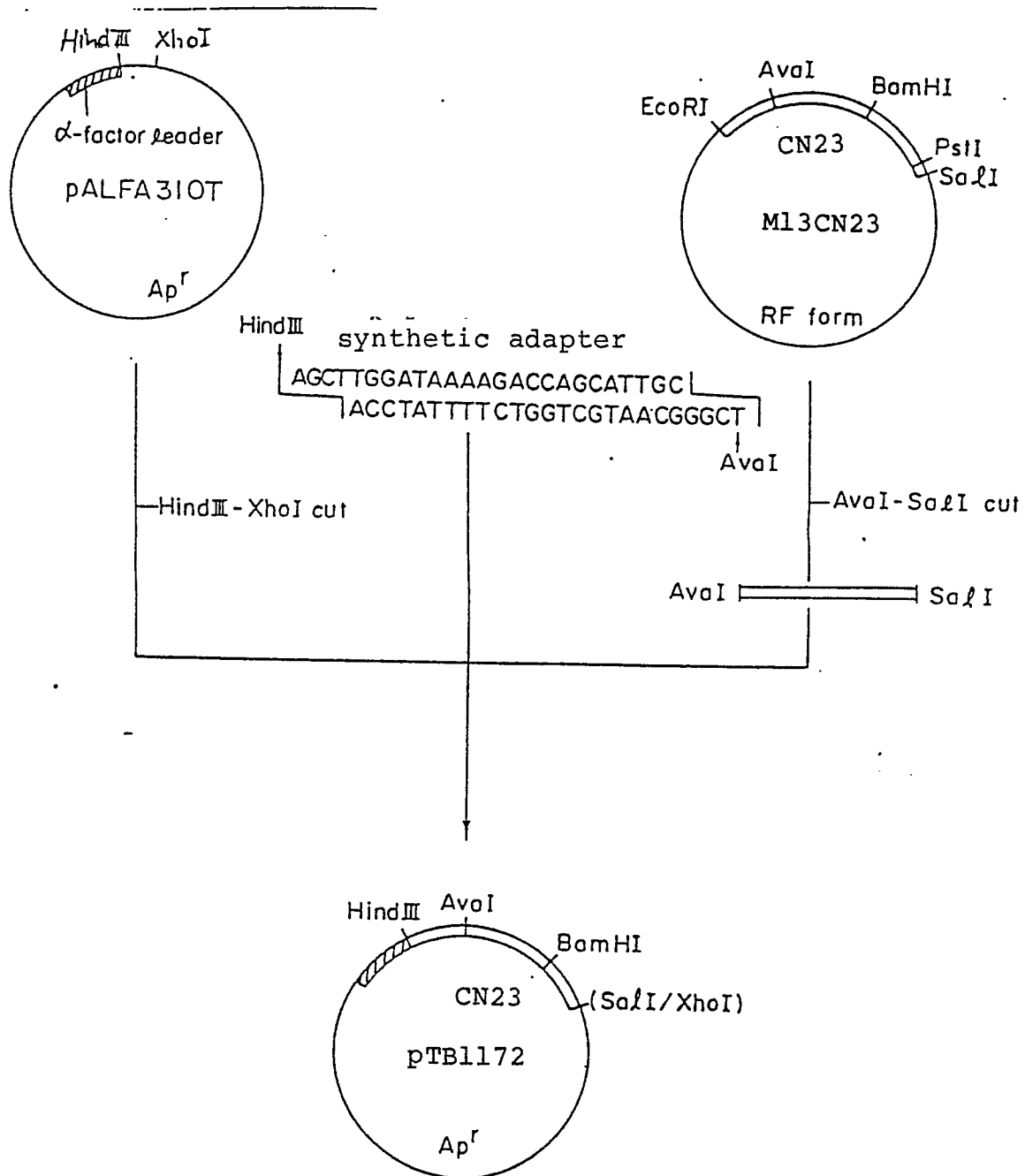


Fig. 11

$\alpha$ -factor ←  
 SerLeuAspLysArg  
 (A) AGCTTGGATAAAAGA\*  
 HindIII

/  
 \*ProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp  
 CCAGCATTGCCCGAGGATGGCGGCAGCGGCCCTTCCCGCCCGCCACTTCAAGGAC

ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg  
 CCCAAGCGGCTGTACTGCAAAACGGGGCTTCTTCTGCGCATCCACCCCGACGGCCGA

ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu  
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ArgGlyValValSerIleLysGlyValAsnAlaThrArgTyrLeuAlaMetLysGluAsp  
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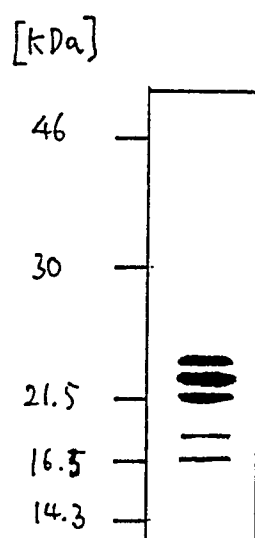
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ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe  
 CGAACTGGGCAGTATAAACTTGGATCCAAACAGGACCTGGGCAGAAAGCTATACTTTT

/<sup>86</sup>  
 LeuProMetSerAlaLysSerTrm  
 CTTCCAAATGCTCTAAGAGCTGA

Fig. 12

A



B

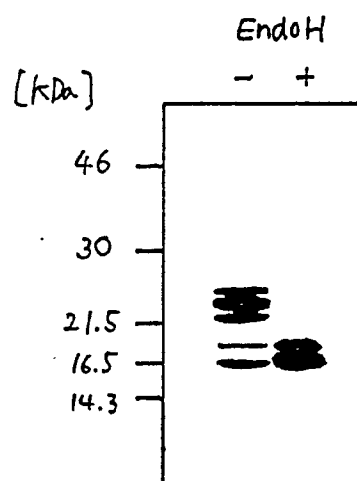
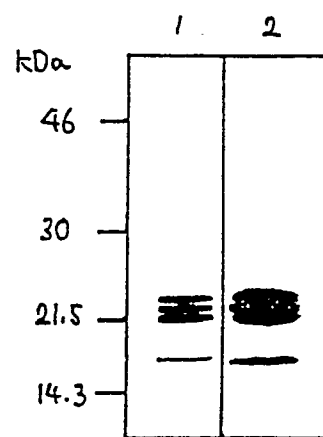




Fig. 13

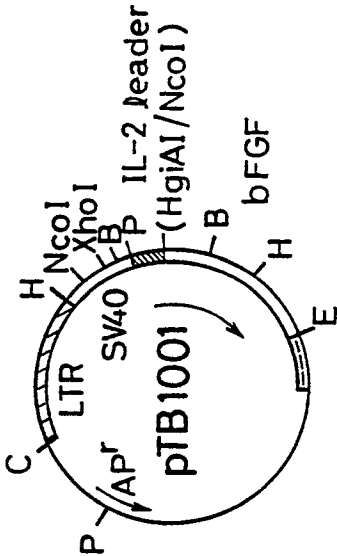


1 : silver staining  
2 : western blotting



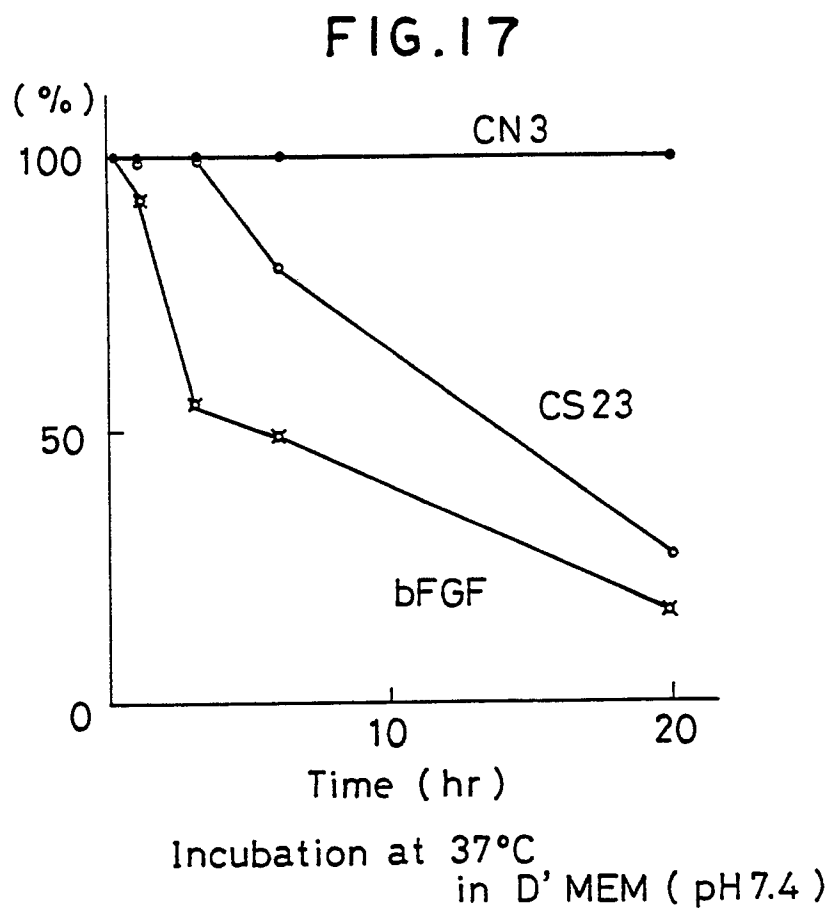
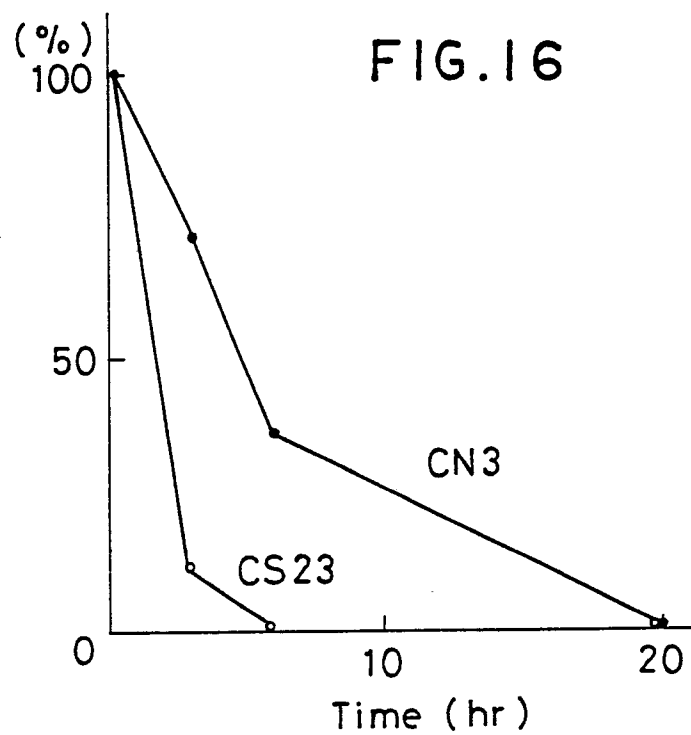
FIG.15

pTB1001



S1 S20 -9 -8 -7 -6 -5 -4 -3 -2 -1 1 2 146  
Met Asn Ser Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Ser  
pTB1001: ---ATG---AAC AGC ATG GCA GCC GGG AGC ATC ACC ACG CTG CCC GCC---AGC TGA---  
(IL-F155) IL-2 bFGF

S1 S20 1 2 146  
Met Asn Ser Pro Ala Ser  
pTB1162: ---ATG---AAC AGC CCC GCC---AGC TGA---  
(IL-F146) IL-2 bFGF





EP 90 10 7737

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP-A-298723 (BIOTECHNOLOGY RESEARCH ASSOCIATES, J.V.) * page 24, line 28 * ---	1, 4, 6-12	C12N15/16 C12P21/02
D,X	EP-A-281822 (TAKEDA CHEMICAL INDUSTRIES, LTD) * figure 54 *and example 39 ---	1, 4-12	
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 138, no. 2, 31 July 1986, DULUTH, MINNESOTA US pages 611 - 617; GIMENEZ-GALLEGO, G. et al: "The complete amino acid sequence of human brain-derived acidic fibroblast growth factor" * page 614; figure 1 * ---	1	
A	BIOL. CHEM. HOPPE-SEYLER vol. 368, no. 9, 1987, page 1060 HOFER, B., et al.: "Modification and introduction of protein glycosylation sites by site-specific mutagenesis: studies on human interleukine-2." * the whole document * -----	1-12	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C12N C12P
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 16 JULY 1990	Examiner ANDRES S.M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... & : member of the same patent family, corresponding document	